UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

EPIDEMIOLOGICAL ANALYSIS OF BLUETONGUE SURVEILLANCE AND VACCINATION DATA IN SOME AUSTRIAN ZONES IN 2008

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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To my parents.

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Epidemiological analysis of bluetongue surveillance and vaccination data in some Austrian zones in 2008

Abstract

Bluetongue virus serotype 8 (BTV-8) was detected in Austria for the first time, in November 2008. Due to outbreaks previously occurred in German regions close to the Austrian border, an active surveillance system was in place and allowed for an early identification of BTV-8 in the country. Mass emergency vaccination was started in the western part of the country in July 2008, due to the inclusion of that area in the protection zone around German outbreaks.

The main objective of this work was to study the occurrence of BTV-8 in Austria in 2008 by i) describing the outbreak in Schärding, ii) comparing the two similar districts with different preventive strategies where BTV was identified - Schärding and Bregenz, iii) evaluating the influence of population dynamics in the duration of vaccinal immunity of cattle from the region of Styria included in the emergency vaccination program, and iv) developing a transmission model for the Styria region.

From the analysis of the BT cases occurred in Schärding it was concluded that the moments of infection were very likely between May and October 2008, considering the optimal temperatures for *Culicoides* abundance that were verified in the region between April and September. The comparison between Schärding and Bregenz, concluded that the former district gathered a higher number of risk factors for disease spread. Higher cattle density in Schärding may have contributed to a higher spread of BTV, whereas the performance of a preventive mass vaccination campaign in Bregenz, most likely contributed for the opposite. It was also found that the proportion of PCR_+ results amongst c-ELISA positive sera was statistically associated to the district of origin. A much lower proportion was observed in Bregenz when compared to Schärding. The analysis of the dynamics of cattle population in Styria resulted in an estimation of 3% year variation in cattle numbers which probably has a negligible effect on the decrease of the HIT in a time-frame of one year, leading to the conclusion that the lost of population immunity to BTV in Styria will be mostly due to the lost of immunity conferred by vaccination that lasts close to one year. The results of the BT transmission model for Styria indicated that the risk of occurrence of secondary infections in the summer months is not negligible, with a maximum estimated R_0 of 2.66.

These studies highlight the importance of epidemiological analysis of available data, using tools like mathematical modeling and GIS in order to understand disease occurrence in animal populations.

Keywords: Bluetonge; Serological surveillance; Preventive mass vaccination; Herd immunity threshold; Mathematical modeling; Basic reproduction number

Análise epidemiológica de dados de vigilância e vacinação de algumas zonas Austríacas em 2008

Resumo

O serótipo 8 do vírus da língua azul (VLA-8) foi detectado na Áustria pela primeira vez em Novembro de 2008. Devido a surtos ocorridos na Alemanha próximo da fronteira Austriaca, um sistema de vigilância activa encontrava-se em curso e identificou o VLA-8 no país. A vacinação massiva de emergência foi iniciada na zona oeste do país em Julho de 2008, devido à inclusão daquela área na zona de protecção à volta dos surtos ocorridos na Alemanha.

O objectivo principal deste trabalho foi estudar a ocorrência do VLA-8 na Áustria em 2008 i) descrevendo o foco ocorrido em Schärding, ii) comparando os dois distritos semelhantes com diferentes estratégias preventivas onde o VLA foi identificado – Schärding e Bregenz, iii) avaliando a influência da dinâmica populacional na duração da imunidade vacinal dos bovinos da região da Styria, e iv) desenvolvendo um modelo de transmissão para a Styria.

Da análise dos casos de LA em Schärding conclui-se que os momentos de infecção se situaram provavelmente entre Maio e Outubro de 2008, considerando as temperaturas óptimas para abundância de Culicoides que aí se verificaram entre Abril e Setembro. A comparação entre Schärding e Bregenz concluiu que Schärding reuniu um maior número de factores de risco para a disseminação da doença. A sua maior densidade de bovinos poderá ter contribuído para uma maior disseminação do VLA, ao passo que a vacinação massiva preventiva em Bregenz, muito provavelmente terá contribuído para o oposto. Foi também observado que a proporção de resultados PCR+ entre soros positivos a c-ELISA estava estatisticamente associada ao distrito de origem, sendo inferior em Bregenz relativamente a Schärding. A variação anual da população de bovinos na Styria foi de 3%, a qual terá um efeito negligenciável no decréscimo da imunidade do efectivo vacinado contra o VLA, sendo esta principalmente devida à perda da imunidade conferida pela vacinação, que dura cerca de um ano. Os resultados do modelo de transmissão de LA para a Styria indicaram que o risco de ocorrência de infecções secundárias nos meses de verão não é negligenciável, com um R0 estimado em 2.66.

Estes estudos sublinham a importância da análise epidemiológica dos dados disponíveis, utilizando ferramentas como a modelação matemática e os sistemas de informação geográfica de modo a compreender a ocorrência de doença em populações animais.

Palavras-chave: Língua azul; Vigilância serológica; Vacinação massiva preventiva; Limiar de imunidade do efectivo; Modelação matemática; Número de reprodução básica

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List of Abbreviations

AGES – Österreichische Agentur für Gesundheit und Ernährungssicherheit

AGID – agar gel immunodiffusion

BMELV – Bundesministerium für Ernährung, Landwirtschaft und Verbrauchershutz

BMGFJ – Bundesministerium für Gesundheit Familie und Jugend

BT – bluetongue

BT-Net – bluetongue network application

BTV – bluetongue virus

BTV-8 – bluetongue virus serotype 8

cDNA – complementary deoxyribonucleic acid

c-ELISA – competitive enzyme-linked immunosorbent assay

CLPs – core-like particles

CTL – cytotoxic T lymphocytes

DIVA – differentiating infected from vaccinated animals

DNA – deoxyribonucleic acid

dsRNA – double-stranded ribonucleic acid

EC – European Commission

ECE – embryonated chicken eggs

EFSA – European Food Safety Authority

EIP – extrinsic incubation period

ELISA – enzyme-linked immunosorbent assay

EU – European Union

GIS – geographical information systems

HIT – herd immunity threshold

i-ELISA – indirect enzyme-linked immunosorbent assay

Ig – immunoglobulin

MAbs – monoclonal antibodies

MLVs – modified live vaccine

MS – Member States

NS – non-structural protein

OIE – Office International des Épizooties

OIE Code – OIE Terrestrial Animal Health Code

OIE Manual – OIE Manual of Diagnostic Tests and Vaccines

OR – Odds ratio

PCR – polymerase chain reaction

p.i. – post infection

p.v. – post vaccination

PZ – protection zone

R_0 – basic reproduction number or transmission value

RA – risk assessment

RNA – ribonucleic acid

RT-PCR – reverse-transcription polymerase chain reaction

RT-rPCR – real time reverse-transcription polymerase chain reaction

RZ – restricted zone

SCFCAH – Standing Committee on the Food Chain and Animal Health

Se – sensitivity

Sp – specificity

SPS Agreement – Agreement on the Application of Sanitary and Phytosanitary Measures

SZ – surveillance zone

VLPs – virus-like particles

VP – viral polymerase

Chapter 1: Introduction

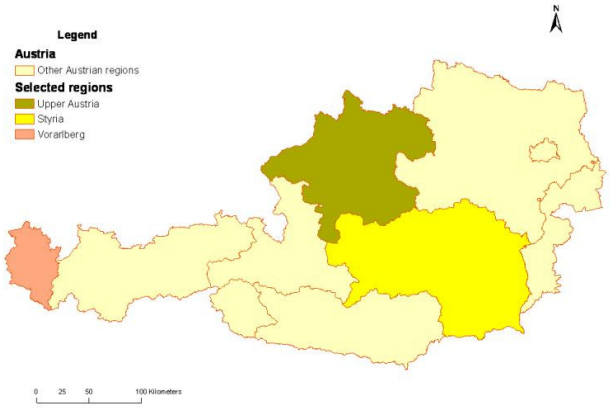
The present dissertation results from the work developed at the final six months training period of the Masters in Veterinary Medicine which was developed in equal periods at the Faculty of Veterinary Medicine of the Technical University of Lisbon (FMV) and the Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), in Austria, under the theme of Bluetongue (BT) surveillance and control.

At FMV, BT literature review and a first approach to data management, descriptive statistics, statistical modeling, risk analysis and geographical information systems was performed in order to develop new epidemiological skills and prepare the work to be developed in Austria. The second half of the training took place at AGES, in Graz, and was dedicated to data gathering and processing regarding the surveillance and control of BT in Austria, as well as to software learning and development of methodologies used in its analysis. Annex II gives an additional description of the contents of each part of the training period including the courses that were attended.

Austria was free from BT until November 2008, when the first occurrences of bluetongue virus serotype 8 (BTV-8) were notified in Schärding, Upper Austria, and later in Bregenz in the region of Vorarlberg. Following the outbreaks in neighboring countries and inside Austria, vaccination was applied in some regions.

The main objective of this work was, therefore, to study the occurrence of bluetongue virus (BTV) in the cattle population in Austria in 2008. Specific goals were i) to describe the outbreak of BTV in Schärding, ii) to compare the two districts where BTV was identified, Schärding and Bregenz, as they applied different preventive strategies, iii) evaluate the influence of population dynamics in the immunity of cattle from the region of Styria which was considered a high risk area for BT occurrence and implemented a preventive vaccination program, iv) to develop a transmission model for the Styria region. The three Austrian regions addressed by the present work are illustrated in Figure 1.

Figure 1 – Austrian regions approached in the present work



In this dissertation a literature review has been made on bluetongue, BT surveillance and monitoring, BT control and eradication, vaccination against BT and on the epidemiological studies, including risk assessment, developed in Europe.

The results of data analysis according with the objectives described above are presented in four separate chapters.

Firstly, in Chapter 3, an analysis of the BT cases occurred in the district of Schärding in late 2008 was undertaken with the aim of identifying the possible origin of those cases and carry out a spatial analysis of the identified cases.

In Chapter 4, the two districts where BTV was notified in 2008, namely Schärding and Bregenz, were compared in an attempt to identify epidemiological differences which could justify the higher number of cases in Schärding. In the same chapter, it was tested the hypothesis that the proportion of cattle sera c-ELISA positive which also resulted positive to polymerase chain reaction (PCR) depended on the district of origin, specifically Schärding or Bregenz.

In Chapter 5, an analysis of the Styria's cattle population dynamics within the time frame of one year was performed, in order to verify if the lost of immunity of the vaccinated population will greatly depend on population changes or mainly on the lost of vaccinal immunity.

Finally, in Chapter 6, the risk of BTV transmission in Styria was assessed, by means of computing the monthly basic reproduction numbers (R_0) for this region.

The last chapter was dedicated to summarize the main conclusions of the analyses developed.

Chapter 2: Literature review

2.1 Bluetongue

Due to its economic impact, bluetongue is a listed disease of the Office International des Epizooties (OIE). Economic losses associated with bluetongue virus infection are caused directly through reductions in productivity and death and indirectly through trade losses due to animal movement restrictions, restrictions on the export of cattle semen and the costs of implementing control and surveillance measures (Schwartz-Cornil et al., 2008).

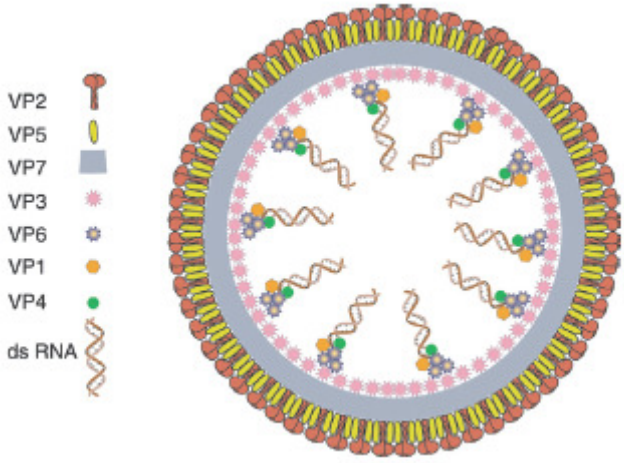
The incursion of BTV into Europe had a considerable negative economic impact mostly due to the ban of ruminant trade between BTV-infected and non-infected areas (Savini, MacLachlan, Sanchez-Vizcaino & Zientara, 2008).

2.1.1 Bluetongue virus

Bluetongue virus is the type-species of the genus *Orbivirus* in the family *Reoviridae*. It was the first virus of domestic animals to be shown to possess a double-stranded ribonucleic acid (dsRNA) genome, initially though to be unique to the genus *Reovirus* (Verwoerd & Erasmus, 1994)

BTV is a non enveloped virus with a genome composed of ten linear segments of dsRNA. These segments are labeled 1 to 10 in descending order of size and each of them codes a different viral protein, respectively the viral polymerases (VP) VP1, VP2, VP3, VP4, VP5, VP6, VP7 and the non-structural proteins (NS) NS1, NS2 and NS3 (Verwoerd & Erasmus, 1994). The 10 dsRNA segments are packaged within a triple layered icosahedral protein capsid. Unlike most single stranded RNA viruses, the Orbiviruses are genetically and antigenically stable throughout infection as point mutations do not appear to arise in vivo at a high frequency. Figure 2 is a representative scheme of BTV structural proteins and dsRNA segments.

Figure 2 – BTV structural proteins and dsRNA segments.



Source of figure: Schwartz-Cornil et al. (2008)

VP2 is responsible for receptor binding, hemagglutination and eliciting serotype-specific neutralizing antibodies. It also has a strong affinity for a component of erythrocytes, an interaction that could be involved in BTV binding to these cells. VP2 is the major determinant of BTV serotype, with a minor role for VP5. Sequencing and phylogenetic comparisons of VP2 also revealed significant variations between strains of the same serotype derived from different geographical areas. These geographical variations define eastern and western VP2 topotypes within individual serotypes. In contrast to VP2, VP5 is significantly more conserved but shows some degree of variation that reflects the geographic origin.

The two major core proteins, VP3 and VP7, constitute a complex which protects the viral dsRNA genome. VP3 and to a lesser extent VP7 are conserved proteins and are hydrophobic in nature. They play an important role in the structural integrity of the virus core and express group-specific antigenic determinants defining several distinct phylogenetic groups. Viral cores are poorly infectious or even non-infectious in different mammalian cells but they are at least 100 fold more infectious for adult *Culicoides* midges. VP7 can mediate attachment and penetration of insect cells in the absence of either VP2 or VP5.

The three minor core proteins VP1, VP4 and VP6 form the transcription complex. VP1 acts as the BTV replicase that synthesizes dsRNA and has an optimal activity between 27 Celsius degrees (ºC) and 37 ºC, allowing efficient replication in both insect and mammalian cells.

The two larger BTV non structural proteins, NS1 and NS2, are the first and second most highly expressed proteins in infected cells, whereas the two closely related minor proteins NS3 and NS3a are barely detectable in mammalian cells. However, NS3 and NS3a are synthesised in much larger amounts in insect cells, suggesting that their role may be primarily related to BTV replication and dissemination within the vector (Schwartz-Cornil et al., 2008).

At present, 24 BTV serotypes have been identified worldwide. The existence of these serotypes, all interrelated in a complex network of cross relationships, has been attributed to antigenic drift and genetic recombination (Verwoerd & Erasmus, 1994). It has been shown that individual BTV genome segments evolve independently of one another by genetic drift during sequential passage of the virus through its ruminant hosts and insect vector. Founder effect also promotes rapid genotypic changes in RNA viruses, as random sampling accidents result in the fixation of specific genotypes to occur. BTV exhibits therefore characteristics of quasispecies evolution as this is not simply a consequence of an accumulation of mutations as the virus replicates (Bonneau, Mullens & Maclachlan, 2001).

2.1.2 Pathogenesis of bluetongue

BTV can affect domestic and wild ruminants. Sheep are the domestic ruminants most affected with BT disease. Cattle usually do not develop severe clinical signs, but only a hypersensitivity reaction due to a rapid accumulation of immunoglobulin (Ig) E antibodies shortly after infection, a transient febrile response, tachypneia, increased lachrymation and salivation and skin inflammation; they are however extremely important in the virus transmission process, acting as reservoirs. Recently, BTV-8 which was responsible for the northern Europe outbreak counteracted these facts, as a large number of infected cattle also developed clinical signs.

The severity of the disease varies with host age and health status and can be exacerbated by concurrent infections as well as by exposure to strong solar radiations, cold or wet conditions. Clinical manifestations may range from subclinical infection or mild disease to acute or even fatal disease. The extreme variability in the clinical manifestation of BT is a feature of the disease. The mortality rate varies between 2 and 30%, and deaths are usually resulting from lung oedema and eventual asphyxia.

Following initial replication of the virus in lymphoid tissues and endothelial cells, viraemia is usually detectable around 3 to 5 days post infection (p.i.) and reaches a peak about seven to eight days p.i.. Viraemia rarely persists for longer than 14 days in sheep and can be prolonged to about 50 days in cattle. (MacLachlan et al., 1994; Verwoerd & Erasmus, 1994; Bonneau, De Maula, Mullens & MacLachlan, 2002). BTV can disseminate via lymph and/or blood; it infects monocytes and T cells, although it is unclear how these cells are involved in the pathogenesis of the virus. BTV can also be detected in the intracellular vesicles of erythrocytes as early as 24 hours p.i. It has been proposed that viral particles associated with erythrocytes are protected from immune clearance. Moreover, the detection of BTV RNA up to 145 days p.i. (Katz, Alstad, Gustafson & Evermann, 1994; Schwartz-Cornil et al., 2008) is

in fact similar to the lifespan of the ruminant erythrocytes, suggesting that these cells may be the critical mechanism that allows cattle to act as natural reservoir hosts of BTV.

Clinical signs in sheep include pyrexia, hyperemia of the buccal and nasal mucosae, increased salivation and lachrymation, licking movements, oedema of the tongue, lips, face and ears; in severe cases the lesions progress to excoriations and erosions, foetid breath resulting from the necrotizing mouth lesions, anorexia, cyanotic tongue ("blue" tongue), dyspnoea, lethargy, rumen stasis and occasionally haemorragic diarrhoea. A panleukopenia reaches its maximum at 7 to 8 days p.i. (preceding the viraemia peak and the associated febrile reaction) and affects all lymphocytes, especially CD8 T-cells.

Gross pathological alterations are characterized by widespread oedema, haemorrhages especially in the lymph nodes, lungs, heart and skeletal muscles, and necrosis of the buccal and nasal mucosae. Microscopic lesions include endothelial hypertrophy, vascular stasis and thrombosis with tissue infarction. Animals that survive acute infection may develop chronic dermatitis, and vesicular and erosive lesions at interdigital and mucosal surfaces.

The incubation period following natural infection is about seven days and the first clinical sign is a febrile reaction (41 to 42 ºC) (Schwartz-Cornil et al., 2008; Verwoerd & Erasmus, 1994)

The pathological changes in skeletal musculature are probably the most important lesions in BT from an economic point of view, as they are associated with loss of condition, weakness and a slow recovery period (Verwoerd & Erasmus, 1994).

2.1.3 Immune response against BTV

Both humoral and cellular immune responses play a role in immunity to BTV.

BTV specific antibodies can confer protection in a serotype specific manner, suggesting an in vivo role for antibody-mediated viral neutralization. However, exactly how antibodies interfere with BTV infection in vivo is unknown. VP2 and VP5 are the only BTV proteins shown to induce neutralizing antibodies. These can protect against the infection by a limited number of other serotypes, associated to similarities of sequences in VP2.

Cellular immune responses mediated by cytotoxic T lymphocytes (CTL) have shown to confer heterotypic, short-lived protection against BTV (Dungu, Gerdes & Smit, 2004a) Studies performed with mice indicated that non structural protein peptides are the predominant source of CTL recognition. In sheep, BTV-specific cross-reactive CTL have been described and CTL lines have been shown to inhibit viral replication in skin fibroblasts infected with homologous and heterologous virus types (Verwoerd & Erasmus, 1994).

Helper T cells are also involved in the immune response to BTV. Major helper serotype-specific determinants are present in VP2 and some in VP5, while major serotype cross-reactive determinants are located within the core structural proteins (VP3 and VP7). CD4 T cells have been suggested as the immune effectors involved in the VP7 induced protection, but their direct functional contribution is still unclear.

2.1.4 Bluetongue epidemiology

BT was first described in South Africa, where it has probably been endemic in wild ruminants from antiquity. It was thought to be confined to the African continent until 1943, when the first outbreak was reported in Cyprus (Verwoerd & Erasmus, 1994). At the moment, BTV is known to be present in all continents except for Antarctica (European Food Safety Authority [EFSA], 2007d).

Early descriptions of BT recognized that the disease was geographically limited and seasonal in its occurrence, which suggested that there was a role of an insect vector in its transmission. The first experimental evidence that BT was transmitted by a *Culicoides* species was obtained in 1944 (Verwoerd & Erasmus, 1994). It is now accepted that the distribution of BTV is dependent on the presence of reservoir and amplifying hosts, and on suitable species of *Culicoides* being present in large enough numbers to effect transmission.

BT oral transmission or transmission by aerosol is highly unlikely and tissues and products from infected animals can be disregarded as a source of infection (Verwoerd & Erasmus, 1994). However, the virus can occasionally be transmitted from vertebrate to vertebrate in seminal fluid and by crossing ruminant placenta. Apparently attenuated vaccine viruses are transmitted more frequently by this routes than are field viruses (Osburn, 1994; Veronesi, Hamblin & Mellor, 2005). Osburn (1994) has described reproductive changes associated with bluetongue as being abortion, malformed lambs or calves, temporary sterility in bulls and rams, and shedding of BTV in semen.

Until recently, BTV serotypes reportedly causing transplacental infections were all assigned to the use of modified live vaccines (MLVs), but during the BTV-8 northern Europe epidemic a significant increase in the incidence of abortions was reported. Since then, several studies were conducted to investigate the occurrence of natural transplacental infection caused by wild-type BTV-8 and to check the immunocompetence of newborns (De Clercq et al., 2008). Evidences were found that BTV-8 field strain was transmitted transplacentally from cow to calf and that vertical transmission could result in healthy looking viraemic calves (Menzies et al., 2008; Santman-Berends, Van Wuijckhuise, Vellema, & Van Rijn, 2009). These calves could play an important role in the epidemiology and in particular in overwintering of BTV-8.

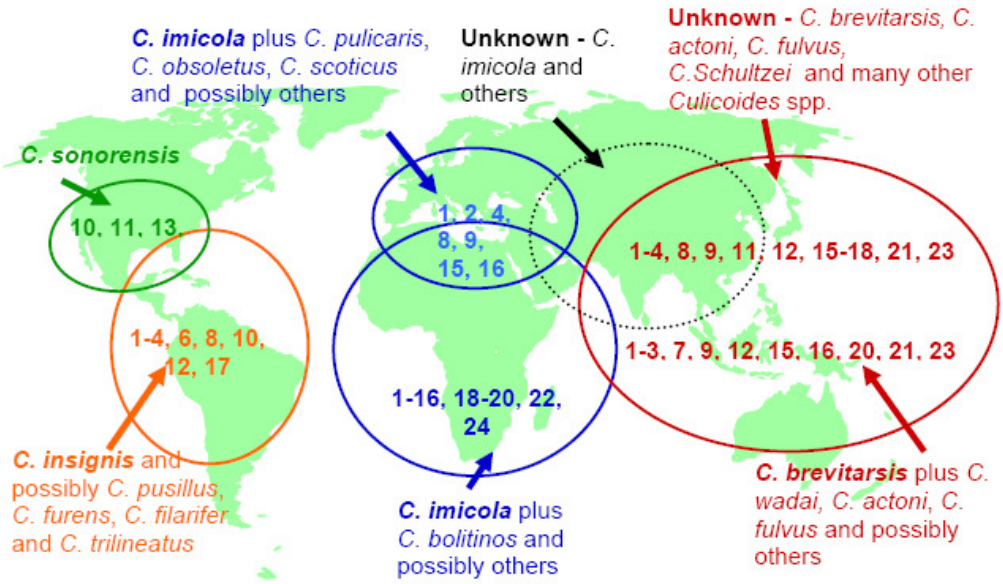
There as been concern that BTV could appear in the uterine environment of viraemic cows at the time of ovulation, which raised the concern about the possibility of BTV transmission to susceptible cows during embryo transfer (Osburn, 1994). However, experimental works have shown that BTV is not able to penetrate the zona pellucida to infect the developing balstocyst, which indicated that embryo transfer can be regarded as a safe procedure, as far as accidental BTV transmission does not occur (Verwoerd & Erasmus, 1994).

BTV may be transmitted throughout the year in areas with mild winters. However, overwintering of the virus in areas with long, cold winters is more difficult to explain, as transovarial transmission of BTV in *Culicoides* spp apparently does not occur. It is possible that cattle are able to act as reservoirs on these circumstances. Cattle have been regarded as being much more important in the epidemiology of BT than other domestic ruminants. They are considered to be more attractive to *Culicoides*, they rarely exhibit clinical signs and they are presumed to support a viraemia of considerably longer duration. It has been suggested that the prolonged viraemia recorded in cattle may enable the virus to bridge vector-free periods and so constitute an overwintering mechanism (Koumbati, Mangana, Nomikou, Mellor & Papadopoulos, 1999).

2.1.5 Bluetongue vectors

Culicoides biting midges are the only significant biological vectors of BTV.

Figure 3 – Global distribution of BTV serotypes and vector species



Source of map: EFSA (2007d); the *Culicoides* species identified in bold are considered the principal vector of BTV in each region (if known).

Around 1210 species of *Culicoides* have been described worldwide; approximately 120 species occur in Europe; only 32 species are considered to be involved in the transmission of BTV.

There are profound differences in the most important species that occur within specific regions of the globe, usually associated with the differences in the BTV serotype occurrence in those regions (Figures 3).

Culicoides in each zone are associated in species complexes with close taxonomic relatives.

In Europe, the species *C. imicola*, *C.obsoletus*, *C. scoticus*, *C. pulicaris* and *C. dewulfi* have been implicated in the transmission of BTV.

Table 1 lists these five species and their relatives, according to subgenus and species complex.

Table 1 – Species complexes of the genus *Culicoides* that play a role in the transmission of BT in Europe.

Subgenus	Species complex	Species
Avaritia	Imicola	***C. imicola***
		C. brevitarsis
		C. bolitinos
	Obsoletus	***C. obsoletus***
		C. scoticus
	Dewulfi	***C. dewulfi***
Culicoides	Pulicaris	***C. pulicaris***

Adapted from EFSA (2007d).

In the Mediterranean basin *C. imicola* is the principal vector of BTV, however within southern Europe *C. pulicaris*, *C. scoticus*, *C. obsoletus* and *C. dewulfi* are also implicated. The latter three species are probably the most important vectors in northern Europe, where *C. imicola* does not occur.

In the last decade there has been the sequential incursion of different BTV serotypes into the Mediterranean basin. This has been perhaps the largest and most continuous outbreak of a *Culicoides*-borne disease recorded. Of particular concern is that its persistence may be causally linked to climate-change.

The efficiency with which vectors transmit BTV varies according to their biting and survival rates, the virus serotype or strain involved, and the ambient temperatures. Genetic differences which control susceptibility to infection and transmission may play a further role. Summarizing, climatic and environmental conditions exert a substantial influence on the transmission of BTV, through both their effect on total vector population size and species

composition as well as replication of BTV within individual insects. Since climatic and environmental conditions vary seasonally and by location, it means that vectorial capacity will also vary in space and time. Such variation must be taken into account when attempting to estimate the potential for BTV to mount an epidemic (EFSA, 2007d).

Influence of climatic conditions

The key events, both in the BTV transmission cycle and in the lifecycle of its vectors, are modulated by temperature and moisture availability (Ward & Carpenter, 1996).

Generally speaking warm temperatures enhance the recruitment, development, activity and survival rates of *Culicoides* vectors. It has been estimated that the optimal temperatures for *C. imicola* range between 18 and 38 ºC whereas for *C. obsoletus* those are between 11 and 27.5 °C. A cold winter or a hot dry summer may substantially reduce vector populations. The transmission potential of BTV is very sensitive to the balance between the extrinsic incubation period (EIP), which decreases at higher temperatures, and daily survival probability of adult *Culicoides,* which is vastly reduced at extremely low and high temperatures.

Moisture availability is the second most important extrinsic variable affecting BTV transmission, as precipitation governs the size and persistence of semi-aquatic breeding sites for the vector (Ward & Carpenter, 1996).

Resuming, increases in temperature (particularly at night-time and in winter) and in precipitation (particularly in summer/autumn) could lead to an increased incidence of BTV transmission.

Regional warming may have increased the importance of Palaearctic vector species (Obsoletus and Pulicaris complexes) in BTV transmission by increasing their population sizes, survival rates and individual susceptibility to the virus. Otherwise, it could have contributed for the incursion of Mediterranean vector species into more northern latitudes. Historically, the BTV has been distributed between latitudes of approximately 40 degrees north and 35 degrees south, but is known to be expanding into the northern hemisphere.

The temperature-controlled late-season decline in vectors' survival suggests that adult *Culicoides* find mid-winter conditions too harsh for them to survive until the following season. However, in northern Europe it has been demonstrated that active adult *Culicoides*, including species of the Obsoletus complex, can be collected in small numbers throughout the winter period (Takken, Verhulst, Scholte, Jacobs, Jongema & Van Lammeren, 2008). It is unknown whether this persistence of adult *Culicoides* through the winter is a recent

phenomenon brought about by the higher average minimum temperatures that are due to climate-change.

Breeding habitats

The larvae of *Culicoides* require moisture for their development and survival. The three principal categories of breeding sites are i) wet soil between aquatic and terrestrial habitats, ii) dung pats of large animals and iii) moist decaying vegetative material.

Midge habitats are largely species-specific, which has a profound influence on the geographical distribution of each species. *C. pulicaris* favours freshwater vegetated swamps (where the water level is above the soil surface); *C. imicola* favours moist (but not waterlogged) nutrient-rich clay soils exposed to full sunlight (Conte, Goffredo, Ippoliti & Meiswinkel, 2007); *C. dewulfi* breeds in cattle dung; *C. obsoletus* is reported to breed in large numbers in deciduous forest leaf litter (Conte et al., 2007) although its ability to exploit a continuum of vegetative habitats that occur on the surface layer of the soil likely explains why it occurs ubiquitously across Europe penetrating also into highly urbanized environments; the closely related *C. scoticus* was reported to breed in fungi.

Considering the two most important vectors of BTV in Europe, namely *C. imicola* and *C. obsoletus*, it is evident that the fragmented distribution of the former within the Mediterranean environment is due to its breeding and environmental preferences. *C. imicola* rears in moisture-retentive nutrient-rich soils exposed to full sunlight, and occurs in areas where the topography is flat or only moderately undulate. Conversely, *C. obsoletus* is more ubiquitously and independent of soil type, occurring in habitats experiencing reduced solar radiation, and penetrating into terrain that is either flat or strongly undulated, and up to elevations of 2000 m (Conte et al., 2007).

Virus-vector interaction

Culicoides become persistently infected with BTV for their entire lifespan after their infection through feeding on an infected ruminant, whereas infection of the ruminant host is transient.

The age-grading of female *Culicoides* is an extremely useful in-field epidemiological tool as it provides valuable information on the level of risk in terms of BTV transmission. It is only from parous and gravid females that BTV may be isolated. Furthermore, a high parous rate is indicative of a high survival rate and increased longevity, which is essential for BTV to be successfully replicated within the vector and for it to be transmitted afterwards to a susceptible host.

The time interval between ingestion of virus and transmission is called the extrinsic incubation period and is temperature-dependent, decreasing in duration as temperature rises; it usually varies between 10 to 14 days (Bonneau et al., 2001). BTV transmission is extremely efficient and in most cases the bite of a single midge will result in the infection of a susceptible host. However, transmission from an infected host to the vector is much less efficient; even at peak viraemia less than 2% of feeding individuals become infected. The number of midges infected after biting a BT viraemic animal depends upon the animal's level of viraemia, the midges' vector competency and the midge attack rate. As the titre of viraemia drops, there is less chance of a biting midge imbibing an infectious dose of virus. Additionally, the ability of a vector to support BTV replication and transmission is controlled by temperature, and it has been shown that as temperature increases, a greater proportion of a vector population becomes susceptible to infection and transmission. The BTV replication rate within the vector increases with temperature, leading to earlier transmission at higher temperatures. The optimal temperature for BTV transmission is between 27 to 30 °C, since within this range most competent vectors survive long enough to transmit and viral polymerase activity peaks.

Transovarial transmission of BTV in *Culicoides* had not been found until recently and was thought not to occur. However, evidence was found indicating that BTV vertical transmission in *Culicoides* may in fact occur (EFSA, 2007d).

Vector-host interaction

Culicoides males feed exclusively on sugar sources such as plant nectar while females feed mainly on blood, although they have been reported to also visit flowers to obtain nectar. Female *Culicoides* congregate where livestock are present, with more congregating around a herd than a single animal. Usually, the larger the group of animals the greater the number of *Culicoides* encountered in their near vicinity (EFSA, 2007d). *C. obsoletus* in particular is found more often at the farms with a higher diversity of animals. The presence of wild ungulates is also considered favorable for *Culicoides* as they provide a natural food source for these insects (Takken et al., 2008).

There are three phases in the events that lead from initial flight to blood feeding: the first phase is dominated by the factors that determine the probability of a midge entering the area containing a suitable host, the second by those factors that lead a midge to alight upon the host and the third by the factors that influence feeding. To enter a target area a midge must be active, and flight activity is influenced primarily by light intensity (more active at low light intensities), the temperature and humidity of the air and wind velocity (more active with wind

speed of less than 1m/second) (Baldet, Delécolle, Cêtre-Sossah, Mathieu, Meiswinkel & Gerbier, 2008). The length of the period of flight activity is determined by the interrelationships of these factors.

It is widely recognized that a mature female midge will feed once every 3 to 5 days; this period may be reduced further at optimal climatic conditions.

It is generally accepted that *Culicoides* practice preferential feeding on cattle. However, this concept may be due to the fact that whereas the surface area available for feeding on a cow is approximately 3 squared meters (m^2) on a sheep it is only 0.09 m^2, because midges are incapable of penetrating the fleece.

There is evidence that different species of *Culicoides* attack different parts of the animal host. *C. obsoletus* attack preferably the belly, especially in the umbilical region, the udder, and also the limbs.

It has been frequently assumed in the past that *Culicoides* are purely exophagic (feed only on animals outdoors) and exophilic (rarely enter buildings). However, during the outbreak of BT in northern Europe, in 2006, *C. obsoletus* and *C. dewulfi* were found to feed inside cattle stables (Baldet et al., 2008). So far, no evidences of endophily and/or endophagy concerning *C. pulicaris* and *C. imicola* were found (EFSA, 2007d).

It is widely assumed that *Culicoides* are generally crepuscular and may continue to be active throughout the night, particularly when nights are overcast, calm and warm. Though, species found at more northerly latitudes display two biting peaks: one after sunrise and the other close to sunset.

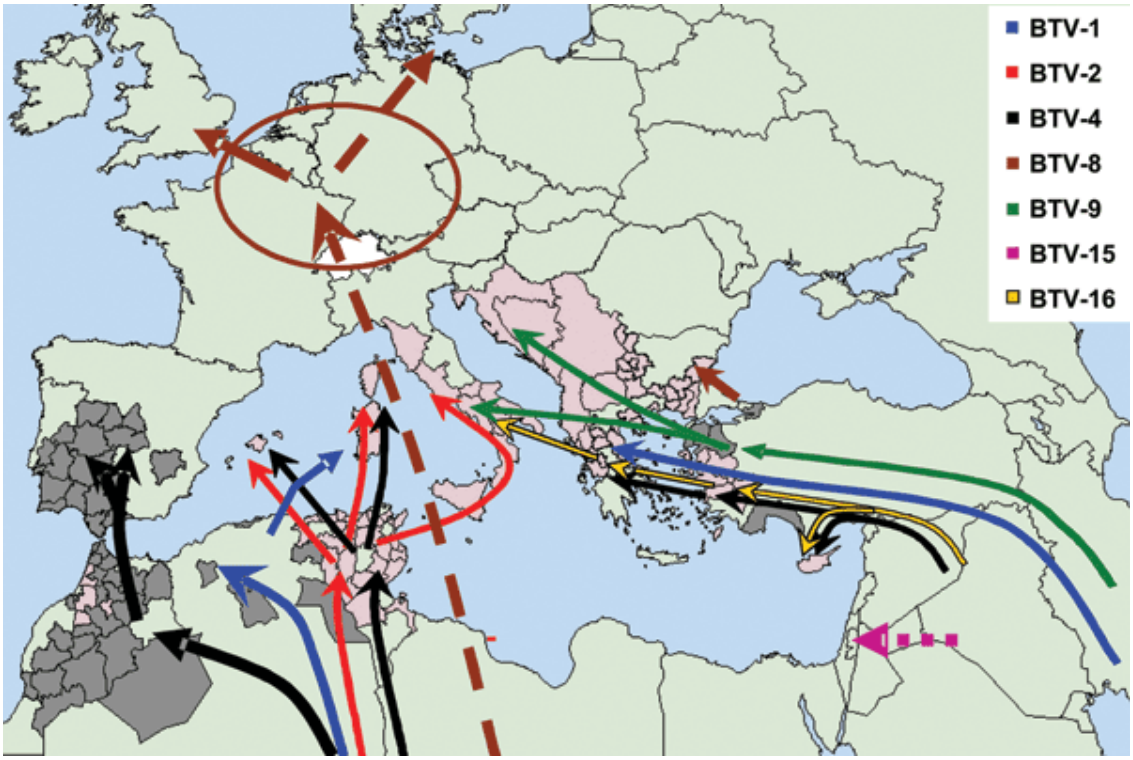
In general *Culicoides* disperse only short distances from their breeding sites, however the potential for considerable movements, transported by wind, is a reality. It has been postulated that in the Mediterranean region *C. imicola* could have been passively carried for distances of over 100 kilometers (km) on winds.

2.1.6 History of bluetongue occurrence in Europe since 1998

Prior to 1998, BTV has on occasion made incursions into Europe, although it has not been able to establish itself permanently in that continent. In 1998 incursions of BTV occurred into countries around the Mediterranean basin.

To date, 6 BTV serotypes have been detected in European territory, namely BTV-1, BTV-2; BTV-4, BTV-8, BTV-9 and BTV-16 (EFSA, 2007d). Figure 4 shows the routes of introduction of each BTV serotype in Europe.

Figure 4 –Routes of introduction of BTV serotypes in Europe.

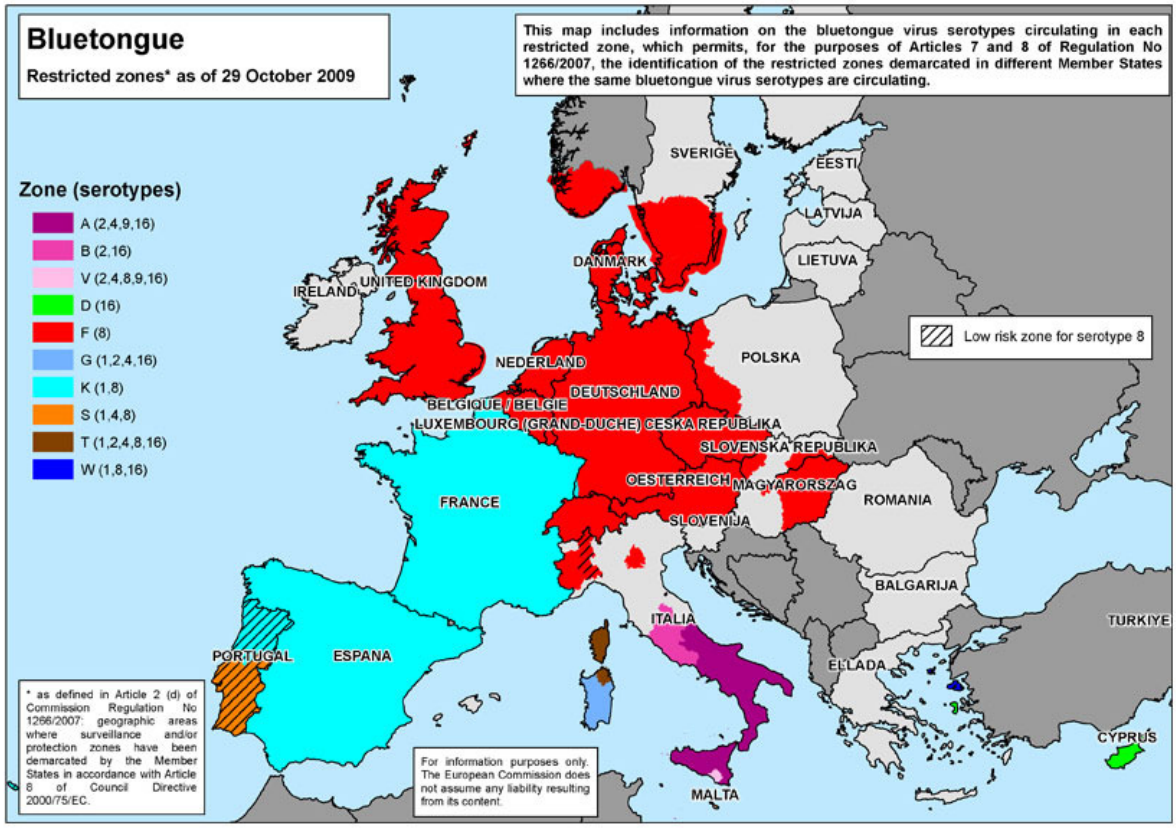


Source of map: Saegerman, Berkvens & Mellor (2008)

Two epidemiologic systems have been identified in the introduction of BTV into mainland Europe: incursions from the Middle East into Greece and the Balkans and from North Africa into Spain, Corsica, Sardinia and the Balearic Islands (Mellor & Wittman, 2002; Saegerman et al., 2008; Roy, Boyce & Noad, 2009). The outbreaks of eastern origin started at the end of 1998 and the outbreaks of south western origin started in the summer of 2000. Serotypes 1, 2, 4, 9 and 16 were involved in these early BT outbreaks in Europe. BTV-8 was first identified in the Netherlands on August 2006 (Saegerman et al., 2008; EFSA, 2007d).

Figure 5 shows the BTV restricted zones in Europe according to identified serotypes, up to October 29th 2009, following the Council Directive 2000/75/EC rules on the implementation of protection and surveillance zones around BTV outbreaks.

Figure 5 – BTV restricted zones in Europe.



Source of map: European Commission [EC], 2009.

BTV-1

There have been two separate introductions of BTV-1 into the Mediterranean region occurred in 2001 and 2006. Phylogenetic analyses showed that the virus strains were distinct, belonging to different 'eastern' and 'western' lineages. The initial strain of BTV-1 in Greece was related to eastern viruses from India and Malaysia and is thought to have entered Europe most probably from Turkey. This strain only persisted for a relatively short period in Greece and did not spread to other European countries. In 2006 BTV-1 was detected in northern Africa and was shown to be clearly distinct from the South African vaccine strain and from the earlier Greek isolates of BTV-1. There were subsequent reports of BTV-1 in Sardinia during 2006, suggesting that the virus had moved northwards into the western Mediterranean islands (Institute for Animal Health [IAH], 2007). In the following year (2007) this strain was detected in Tunisia, France, Italy, Portugal and Spain (Istituto Zooprofilattico Sperimentale [IZS], 2008).

BTV-2

BT outbreaks were recorded in Tunisia during 2000 and the virus involved was identified as BTV-2, belonging to a western lineage. This virus was related to other BTV-2 strains from Nigeria and South Africa, although it was distinct from the South African BTV-2 vaccine strain**.** BTV-2 reached the Italian island of Sardinia in 2000 and later spread into Sicily, the French island of Corsica and the Spanish Balearic islands of Menorca and Mallorca. In 2001, BTV-2 spread across south-west mainland Italy. There is evidence that some of the isolates of BTV-2 from mainland Italy were similar to the BTV-2 vaccine strain that was also used in the region, indicating that the vaccine strain can be transmitted in the field (Ferrari et al., 2005).

BTV-4

Widespread bluetongue outbreaks in Greece during 1999 and 2000 were caused by a strain of BTV-4. Phylogenetic analyses demonstrated that the virus was similar to earlier isolates from Cyprus and Turkey**,** which suggests that the BTV-4 strain which invaded Greece and the eastern Mediterranean region since 1999 belonged to a western lineage and was distinct from the South African vaccine strain. In 2003, BTV-4 was detected in the western Mediterranean islands, including Menorca. This virus strain is believed to have entered Europe from north Africa. The same strain of BTV-4 was subsequently isolated in Morocco and then spread across the Strait of Gibraltar into mainland Spain. In 2004, the virus spread within the Iberian Peninsula, where it persisted through 2005 (Mellor & Wittman, 2002; Gómez-Tejedor, 2004; Mellor, Carpenter, Harrup, Baylis & Mertens, 2008).

BTV-9

The first serotype to arrive in Europe in 1998 was BTV-9. The strain analysis showed that this was an ‘eastern’ virus, related to strains from Indonesia and Australia. BTV-9 was first reported in the Greek islands, and then in the summer of 1999, in Turkey and Bulgaria. In 2001, the disease advanced westwards and northwards, reaching central and north-west mainland Greece, and neighboring Balkan states (southern Bulgaria, Kosovo, Montenegro and Bosnia). It also appears likely that BTV-9 in Italy originated from the same initial source although an isolate of BTV-9 was made in Sicily which was identical to the BTV-9 vaccine strain (IAH, 2007). In 2002 Greece was reported free from BTV but disease due to BTV-9 was still widely reported from the Balkans (Mellor, 2004).

BTV-15

A sample of a virus-isolate that was made in Israel from outbreaks of disease in 2006 was typed as BTV-15. Subsequent phylogenetic analyses confirmed the virus serotype and indicated that it belonged to a western lineage. The number of available BTV-15 isolates is very limited, so it is difficult to be more precise about the origins of this virus strain. However, the existing data suggest that it is new to the region and may therefore represent a further threat to Europe in the future (IAH, 2007).

BTV-16

The initial European strain of BTV-16 was isolated in Greece during 1999. Phylogenetic analysis showed that this virus was from an eastern lineage and was very similar to strains of BTV-16 from Turkey; the same analyses have also shown that this European field strain was closely related, although distinct from the BTV-16 vaccine strain. Therefore its appearance in Europe may be related to the use of live BTV-16 for several years as part of an annual vaccination campaign in Israel. After its arrival in Europe the virus spread eastwards. In 2004, BTV-16 caused outbreaks in Sardinia and in Cyprus. In the first case the strain involved was identical to the BTV-16 vaccine strain that was used in mainland Italy, suggesting that this was a 'vaccine' outbreak and was not caused by the European field strain, whereas the BTV-16 strain identified in Cyprus was similar to the earlier eastern Mediterranean isolates (IAH, 2007).

BTV-8

In August 2006, BT was recognized for the first time in northern Europe, in the Netherlands, being considered as an emergence disease in this part of the continent.

The disease was subsequently detected in Belgium, Luxemburg, Germany and north-eastern France. Sequence analyses demonstrated that the virus was from a western lineage from sub Saharan Africa, but distinct from the BTV-8 vaccine strain. It is uncertain exactly how BTV-8 arrived in northern Europe (EFSA, 2007d), but the absence of BTV-8 outbreaks in southern Europe suggests that it did not involve simple linear extension of earlier outbreaks and is likely to reflect a distinct entry route and mechanism (IAH, 2007).

The recrudescence of BTV-8 in northern France, the Netherlands, Belgium, Luxembourg and Germany in 2007, and also the emergence of BTV-8 in other European countries (Czech Republic, Denmark, Switzerland and United Kingdom), suggests that BTV is able to survive regularly between vector seasons and become endemic to northern Europe (Saegerman et al., 2008). BTV-8 occurrence was confirmed for the first time in Spain, in January 2008, and in

Italy by March (IZS, 2008). Late in 2008 BTV-8 was identified in Sweden and in Austria, and by February 2009 it reached Norway.
The present work was focused on the BTV-8 occurrence in Austria. Therefore, a deeper review on this serotype was performed.

2.1.7 Appropriate conditions for BTV-8 spread and establishing

In 2006, European Food Safety Authority (EFSA) was requested by the European Commission (EC) to carry out a global epidemiological analysis of the ongoing epidemic of BTV-8 in northern Europe. In 2007, EFSA published a report gathering information about the 2006 BTV-8 epidemic, specifically its time-space characteristics, information characterizing within-herd virus spread, information on factors favoring virus establishment, factors that can be used to predict virus persistence, elements that may influence short-distance spread and factors affecting long-distance spread (EFSA, 2007a).

Space-time characteristics of BTV-8 epidemics

Space and time exploratory analysis were used to test for the presence of spatial and temporal patterns. Mapping of the BTV-8 outbreaks reported in Belgium, France, Germany and Netherlands, indicated that infection spread in an east-west and southward direction.
The temporal pattern of BTV-8 outbreaks assumed a bi-modal shape, which was explained by the estimated time lag of 4 weeks between the reduction in transmission frequency and the reduction in the associated number of disease notifications, due to the sum of the incubation period in the insect, the delay between biting meals, the incubation period in the animals and the delay between appearance of clinical signs and disease notification (EFSA, 2007a).

Within-herd spread

Data from whole herd sampling suggested that in infected cattle herds, including those where clinical signs are not observed, there was a high proportion of PCR- and sero-positive animals, while in infected sheep herds showing clear clinical signs, the proportion of PCR- and sero-positive animals was low (EFSA, 2007a).
These facts encourage the thought that BTV spread within cattle herds might be more significant than within sheep herds, but the most important conclusion to draw is regarding the appropriate monitoring/surveillance system to apply to both kinds of herd.
The possible role of wild ruminant species in the epidemiology of BTV cannot be excluded, as they are considered potential reservoirs for the virus. In Germany, positive serological samples from different wildlife species were found in the area where livestock was most

severely affected. The proximity of livestock herds to wild ruminant habitats is therefore important to be considered.

Vectors

The *Culicoides* species endemic to the Palaearctic region have increased their importance in the establishment and spread of BTV as the virus started to appear in northern Europe. This conclusion derives from the following findings: i) *C. imicola* was not found amongst the *Culicoides* collected during the BTV-8 epidemic; ii) considering the rapid spread of BTV-8, it apparently did not require an adaptative phase to Palaearctic vector species; iii) more than one Palaearctic species of *Culicoides* were involved in the BTV outbreak across northern Europe (*C. dewulfi*, *C. obsoletus* and *C. scoticus*); iv) the Obsoletus complex (*C. obsoletus* and *C. scoticus*) was found to be exceptionally widespread in livestock farms in northern Europe; v) the Obsoletus complex as well as *C. dewulfi* showed to have high parity rates of 40% (40% of the individuals captured comprised older females indicating that their survival rate was high and that they were feeding repeatedly on hosts) (EFSA, 2007a).

During the BTV-8 epidemics analysis it was also found that vector species *C. obsoletus* and *C. dewulfi* entered animal housing, especially later in the season when night-time temperatures began to drop to single digits. In light of such findings additional points of concern emerged, such as i) the possibility of indoor vector breeding, ii) the overwintering of BTV-infected late-season adult *Culicoides* and spring recrudescence of BTV transmission cycle and iii) the lack of feasible solutions for the protection of housed animals from vector attack (EFSA, 2007a).

Meteorological conditions

The daily variation of the number of *Culicoides* captured during the BTV-8 epidemic was found to be correlated with prevailing temperatures, for all vector species involved. The lag time between a change in temperature and the change in the reported number of outbreaks was estimated as 4 weeks. The presence of BTV-8 was favored in averagely warmer locations, where temperatures varied less throughout the year and rose quickly in spring.

Although temporal distribution of rainfall and the occurrence of BTV-8 cases did not show association, humidity, which could better describe the influence of moisture on the dynamics of vector population, could not be investigated regarding the BTV-8 epidemic in northern Europe due do data limitations (EFSA, 2007a).

Animal densities and environmental factors

Landscape elements also influence the spatial distribution of BTV. A strong association between temperature and altitude, with hilly areas showing cooler temperatures compared to lower areas, contributes to the role of mountains in limiting the spread of BT infection. BTV-8 cases in northern Europe epidemic showed an upper altitude limit of approximately 650 m. However, the absence of BTV in hilly areas may also be explained by the lower cattle and sheep densities or by the higher proportion of forest areas in such zones. In fact, land-cover is also an important influencing factor in the BTV vector distribution. Areas with a high proportion of urbanization apparently limit the spread of BTV due to the lower density of domestic ruminants. Concerning forests, it seems possible that different vector species show preference for areas with different forest-cover percentages. For instance, in affected areas with low forest-cover percentage and high values of cattle density, *C. dewulfi* likely plays a more important role in the transmission of BTV, whereas in areas with high percentage of forest, *C. obsoletus* is probably the prevailing vector species (EFSA, 2007a).

Factors affecting short-distance spread

During the BTV-8 epidemic in northern Europe, the observed speed of local spread was about 2 km per day (approximately 15 km per week).

There was no significant statistical association between the implementation of the first control measures and the incidence of new BTV-8 infections. However, it is important to stress that this result must be interpreted with care, firstly because it concerns a vector-borne disease and it is difficult to limit local vector movement and secondly because the consequences of not taking control measures at all could not be assessed in order to compare with the mentioned findings. Not taking control measures at all possibly, if not most likely, would have resulted in even wider spread of BTV-8 (EFSA, 2007a; Mintiens et al., 2008).

Factors affecting long-distance spread

BTV-8 epidemic spread into new areas was predominantly horizontal in an east-west direction, and had a limited south spread and a very limited north spread. Density of wind events is so far the only variable identified which describes the observed spatial spread of outbreaks in such a comprehensive way. Movement of infected domestic or wild ruminants or infected ruminant-live products from an infected area may have also contributed to the spread of BTV-8, since it was found a significant positive relationship between the total number of BT cases occurred in a municipality by the end of the epidemic and the total number of

ruminants that were introduced in that municipality prior to and during the epidemic period (EFSA, 2007a).

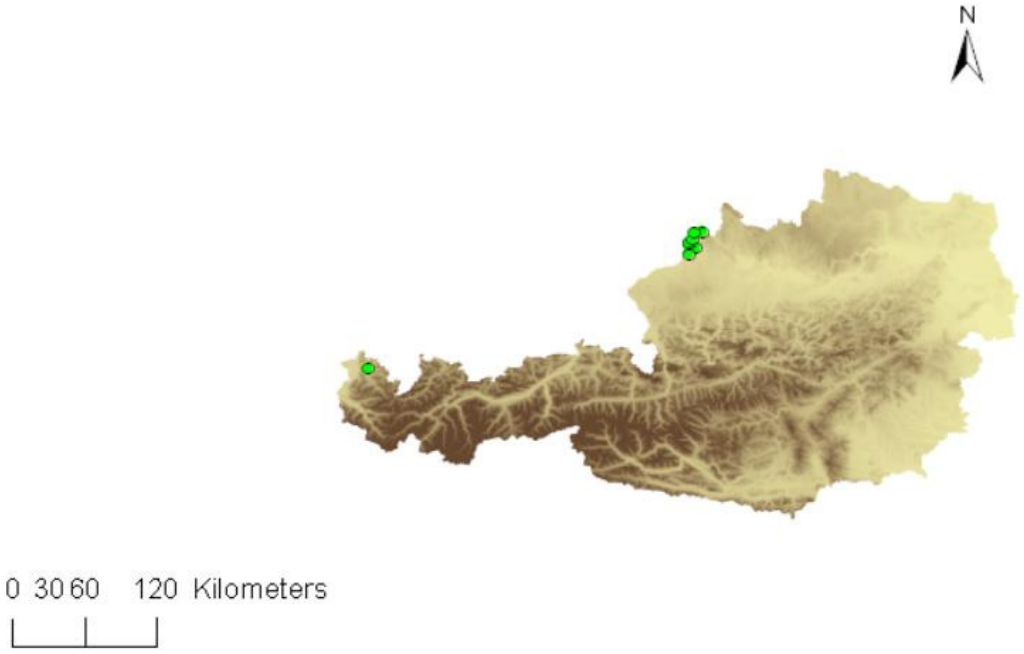
2.1.8 Bluetongue occurrence in Austria

BTV-8 was introduced in Austria in November 2008, in the sequence of the northern Europe outbreak of this serotype which has started in 2006. BT is an emergence disease in this country as this was the first BTV incursion ever recorded.

The first outbreak occurred in the north-eastern region of Upper Austria, close to the border to Germany and Czech Republic. A single district, Schärding, was involved in that outbreak. The first case was confirmed within a routine screening on infectious bovine rhinotracheitis and infectious pustular vulvovaginitis, on a small farm with a mixed animal population.

A month later, BTV-8 was first detected outside of Schärding, in the district of Bregenz, in the western region of Vorarlberg. Between November and December 2008 the overall number of BTV-8 cases was 10 in Schärding and 1 in Bregenz (Figure 6).

Figure 6 – BTV-8 cases occurred between November and December 2008, in Austria.



2.2 Surveillance and monitoring of Bluetongue

According to the Commission Regulation (EC) nº 1266/2007, a case of bluetongue means an animal that meets one of the following requirements:

> i) it presents clinical signs consistent with the presence of bluetongue;
>
> ii) it is a sentinel animal that had showed negative serological results in a previous test and has seroconverted from negative to positive for antibodies to at least one bluetongue serotype since that test;
>
> iii) it is an animal from which the bluetongue virus has been isolated and identified as such;
>
> iv) it is an animal which has tested positive to bluetongue serological tests or from which viral antigen or viral ribonucleic acid (RNA) specific to one or more of the bluetongue serotypes has been identified (Commission Regulation (EC) nº 1266/2007).

Additionally to meeting one of these requirements, there must be epidemiological data indicating that the clinical signs or the laboratory results are the consequence of virus circulation in the holdings and not the result of the introduction of vaccinated or seropositive animals from restricted zones (Commission Regulation (EC) nº 1266/2007).

2.2.1 Surveillance strategies

According to the OIE Terrestrial Animal Health Code (OIE Code) chapter dedicated to bluetongue, the purpose of surveillance is the detection of virus circulation in a country or zone, reason why it deals with all evidences of infection with BTV, independently of the occurrence or absence of clinical signs of the disease (Office International des Épizooties [OIE], 2008c). Therefore, the BT case definition present in the OIE code and established by the Commission Regulation (EC) nº 1266/2007 can be one of the following:

> 1. BTV has been isolated and identified as such from an animal or a product derived from that animal, or
>
> 2. viral antigen or viral ribonucleic acid (RNA) specific to one or more of the serotypes of BTV has been identified in samples from one or more animals showing clinical signs consistent with BT, or epidemiologically linked to a confirmed or suspected case, or giving cause for suspicion of previous association or contact with BTV, or
>
> 3. antibodies to structural or nonstructural proteins of BTV that are not a consequence of vaccination have been identified in one or more animals that either show clinical signs consistent with BT, or epidemiologically linked to a confirmed or suspected case, or giving cause for suspicion of previous association or contact with BTV (OIE, 2008c).

Surveillance can be divided into four categories according to its methodology, namely clinical, vector, serological and virological surveillance. They have different applications regarding BTV infection. For instance, clinical surveillance may be effective in sheep while serological surveillance is more appropriate in cattle (OIE, 2008c).

Clinical surveillance

Clinical surveillance aims at the detection of clinical signs of BT at the herd level. Every suspect animal should be further confirmed by laboratory testing and the absence of clinical disease does not prove freedom from BTV (Cagienard et al., 2004). However, the importance of clinical surveillance should not be underestimated. Diseased animals are detected more rapidly by clinical examination than by serological or virological tests, which makes clinical evaluation more reliable than those methods in detecting incursion of BTV in the beginning of an epidemic. As the disease becomes established in a country or zone, clinical surveillance looses its importance towards serological surveillance (Giovannini et al., 2004a).

Vector surveillance

Vector surveillance, by determining the species of *Culicoides* present in an area, and their respective seasonal occurrence and abundance, aims to define high, medium and low-risk areas and local details of seasonality for the presence of competent vectors of BTV. Long term vector surveillance allows the evaluation of the effectiveness of vector suppression measures. Although it is advised that the operation sites should have the same location as sentinel groups, vector surveillance should not however be used alone as a means of detecting the circulation of BTV (OIE, 2008c).

Serological surveillance

Serological testing of ruminants is one of the most effective methods of detecting the presence of BTV in a country or zone and it may be required for i) serological surveillance, ii) facilitate safe international animal movement, iii) monitoring vaccination campaigns or iv) serotype identification of field BTV strains (Hamblin, 2004).

Besides the serological surveys, serological surveillance usually relies on the sentinel animal programmes. Sentinel units are particularly useful for surveillance of diseases with a strong spatial component (OIE, 2008b). Sentinel animals are therefore the OIE's preferred strategy for BTV surveillance; "They comprise groups of unexposed animals managed at fixed locations and sampled regularly to detect new BTV infections" (OIE, 2008c). Cattle is the most appropriate species to include in a sentinel group, since the collected data have been, to

date, showing that there is a trend suggesting a high proportion of cattle to be sero-positive in infected cattle herds and a small proportion of sero-positive sheep in infected sheep herds. A clinical surveillance system is therefore more appropriate to sheep flocks whereas serological surveillance appears to be the most effective approach for cattle herds (EFSA, 2007a). But besides from species, the selected animals should also be of similar age and susceptibility to BTV. The effect of secondary factors that may influence infection by BTV should be monitored and controlled so that the only feature distinguishing groups of sentinels is their geographical location.

Serological surveillance demonstrates BTV circulation by testing of samples for antibodies against BTV, using the serological tests prescribed in the 2008 OIE Manual of Diagnostic Tests and Vaccines (OIE Manual).

A positive serological test may result from "a) natural infection with BTV, b) vaccination against BTV, c) maternal antibodies, d) positive results due to lack of specificity of the test" (OIE, 2008c).

Both serotype and serogroup-specific antibodies are produced during infection with BTV. Serogroup-specific antibodies may protect the animal against serotypes to which it hasn't been exposed.

The serological tests for BTV described in the OIE manual are the complement fixation, the agar gel immunodiffusion (AGID) and the competitive enzyme-linked immunosorbent assay (c-ELISA). All of these tests are serogroup-specific, detecting only serogroup-specific antibodies (Hamblin, 2004).

Complement fixation was widely used until 1982 when it was replaced by the AGID test. The last one has been the standard test procedure for international movement of ruminants, and it is still prescribed for international trade in the OIE Manual, but it proved to have lack of specificity in that it could detect antibodies to other Orbiviruses, specifically to the epizootic haemorragic disease virus (OIE, 2008a; Koumbati et al., 1999). The development of enzyme-linked immunosorbent assay (ELISA) based procedures was therefore encouraged and c-ELISA is presently the preferred prescribed test for serological surveillance and international trade. c-ELISA tests detect BTV-specific antibodies and do not detect cross-reacting antibodies to other Orbiviruses. Its specificity results from the use of BT serogroup-reactive monoclonal antibodies (MAbs) which bind to the amino-terminal region of the major core protein VP7 and compete with the test sera antibodies for binding to the antigen of the test (OIE, 2008a). c-ELISA has proven to be more sensitive in detecting BTV antibodies than the AGID test (Reddington, Reddington & MacLachlan, 1991; Koumbati et al., 1999; Shringi & Shringi, 2005) and the estimated relative sensitivity and specificity of c-ELISA when

compared to the former were of 100% and 77% respectively (Shringi & Shringi, 2005). c-ELISA further has the advantage of requiring significantly less time to run.

Amongst the Third International Symposium on Bluetongue recommendations was that the AGID test should remain in the OIE manual but not be a prescribed test for international trade, since c-ELISA is currently the standard technology (OIE, 2003).

Even though c-ELISA represents the OIE's prescribed serological test for the diagnostic of BTV infection, it frequently fails the diagnostic of recent infections. In a recent viral infection, the first immunoglobulin antibody to appear in the blood is of the IgM class (earlier than 10 days post infection). IgG antibodies are produced later in infection. c-ELISA is not able to differentiate between IgM and IgG antibodies and the last ones appear to have more affinity for the epitope to which the Mabs bind in c-ELISA. Therefore, in an early infection c-ELISA might give rise to false negative results (Zhou, Ridd, Riva, Fernando & Clavijo, 2001). In 2001, an IgM-capture ELISA was developed for the detection of recent infection of BTV in cattle and compared with c-ELISA. It was concluded that it was a sensitive diagnostic method for the detection of recent infection in cattle (Zhou et al., 2001). However, performance evaluation with field samples was still required as well as the development of a similar test to detect early infection in sheep. Presently, IgM-capture ELISA is available for use with all domestic ruminants (Id vet- Innovative diagnostic kits, 2009) and according to OIE recommendations, it would not only provide information on recent infection status but also allow for the determination of the correlation between IgM antibodies and the duration of viraemia in infected animals (OIE, 2003).

Virological surveillance

Virological surveillance allows for the isolation and/or genetic analysis of BTV from infected animals and its main benefit lies on the provision of information on the serotype and genetic characteristics of the viruses concerned. It can be conducted to "i) identify virus circulation in at risk populations, ii) confirm clinically suspect cases, iii) follow up positive serological results, iv) better characterize the genotype of circulating virus in a country or zone" (OIE, 2008c).

Similarly to the serological tests, virological tests for detection of BTV are also described in the OIE Manual, where they are divided into three categories: virus isolation, immunological methods and reverse-transcription polymerase chain reaction (RT-PCR).

Virus isolation may be performed *in vitro* or *in vivo* (isolation in sheep). The currently most practical method of virus isolation is performed in embryonated chicken eggs (ECE) which efficiency is at this time considered to be significantly higher than that achieved with cell

cultures. The isolated virus resulting from the above mentioned techniques can be further identified directly by antigen-capture ELISA or RT-PCR, or indirectly by immunological methods.

The immunological methods can be subdivided in serogrouping tests and serotyping serum neutralization tests. Serogrouping Orbiviruses isolates is based on their reactivity with specific MAbs antisera that detect BTV proteins such as VP7. Serum neutralization tests are type specific for the 24 BTV serotypes currently recognized and are probably the most used tests in type-specific assays (Hamblin, 2004).

The third category of virological tests comprises RT-PCR techniques, the currently OIE prescribed virological tests for international trade. These techniques have allowed the rapid detection of BTV nucleic acid in blood and other tissues of infected animals, therefore also allowing the serogrouping and serotyping of samples within a few days, oppositely to the traditional approach of virus isolation followed by virus identification, which required up to four weeks (OIE, 2008a). Primers derived from the more highly conserved genes, as those from proteins VP3, VP7 and NS1, may be used for serogrouping, while primers from VP2 gene sequences provide information on virus serotype. However, amongst the same serotype, the nucleic acid sequence of cognate BTV genes may differ with the geographical area where it was isolated from. This information has provided the opportunity to demonstrate the potential geographical origin of virus isolates, a process called genotyping or topotyping, considering however that "the relationship between sequence and geographical origin may not be straightforward" (OIE, 2008a). Still, one of the OIE's recommendations is that the neutralization-based virus serotyping system be replaced by a genetic typing system (OIE, 2003).

RT-PCR assays have proven to detect very small numbers of nucleic acid molecules even after the virus can be detected by virus isolation.This characteristic poses two issues about RT-PCR interpretation. Firstly, it becomes exquisitely sensitive to contamination by extraneous nucleic acids, and secondly a positive result does not necessarily match an active infection, since it detects nucleic acid in the sample long after the clearance of the infectious virus from the animal (OIE, 2008c). It is currently assumed that cattle whose blood samples were positive to RT-PCR analysis but negative to virus isolation had no importance in the epidemiology of BTV infection (MacLachlan et al., 1994; Bonneau et al., 2002).

The possibility of false positives and false negatives occurrence is also an important issue regarding RT-PCR. False positives may be due to sample contamination and false negatives due to poor sample quality or inappropriate primers (OIE, 2008a).

According to Shaw et al. (2007), the majority of primer sets target BTV genome segments 5 and 7, which code respectively viral proteins NS1 and VP7. Both are highly conserved proteins across the BTV species and VP7 in the major serogroup-specific antigen. Therefore, the use of these primers in RT-PCR confers a high level of specificity to the test.

The RT-PCR test described in the OIE Manual is a conventional gel-based nested RT-PCR which targets the segment 5 of BTV. The assay of a conventional RT-PCR test involves three sequential procedures: i) extraction of BTV RNA from sample, ii) denaturation of viral double-stranded RNA and reverse transcription to generate complementary deoxyribonucleic acid (cDNA), which is then amplified and iii) analysis of the RT-PCR product by gel electrophoresis. The nested procedure involves two sets of primers, used in two successive runs of PCR. This method minimizes the probability of occurrence of primers binding to incorrect regions of the cDNA, giving unexpected products, which frequently happens in conventional RT-PCR tests.

Real time RT-PCR (RT-rPCR), also called quantitative RT-PCR enables both detection and quantification of a specific sequence in a cDNA sample. The amplified cDNA is quantified (by the number of its copies) in real time after each amplification cycle.

Real-time assays present some advantages oppositely to conventional RT-PCR tests. Firstly, the use of RT-rPCR avoids the time consuming gel electrophoresis step making it faster to perform than the conventional tests, secondly they are well suited to analyze multiple samples an thirdly they amplify the target cDNA within a closed-tube format, which significantly reduces the risk of cross contamination that could generate a false positive result. Although these advantages are attributed to RT-rPCR, Batten et al. (2008) concluded that the nested RT-PCR recommended in the OIE Manual showed comparable sensitivity to the most sensitive RT-rPCR assays; besides, RT-rPCR technology requires more expensive equipment compared to conventional RT-PCR (OIE, 2003).

After the incursion of BTV into Europe, many studies have been carried out in order to improve the RT-PCR and RT-rPCR tests (Orrù, Ferrando, Meloni, Liciardi, Savini & De Santis, 2006; Shaw et al., 2007; Toussaint, Sailleau, Breard, Zientara and De Clercq, 2007) and some of the resulting improvements were already introduced in national virological surveillance plans.

Despite of the methodology used, the surveillance strategy might be active or passive (according to the means by which data are collected), random or targeted. Random surveillance consists on population-based surveys and is usually conducted using serological tests, which positive results should be further followed up with virological methods. In random surveys, the level of confidence of the results is determined by the sample size and

the expected prevalence of infection in the population (OIE, 2008c). Targeted surveillance is based on non-random surveillance activities and includes the previously mentioned sentinel units. Virological and serological methods may be used concurrently to define the BTV status of targeted populations (OIE, 2008b; 2008c).

The most complete surveillance program should be composed of random and targeted approaches using clinical, serological and virological methods in all domestic ruminants. Active and passive surveillance should be ongoing. However, there are different strategies which give preference for one or more of those methodologies. The type of surveillance applied depends on the desired outputs, specifically, demonstrating absence of disease or infection, determining the occurrence or distribution of disease or infection, or early detecting emerging diseases (OIE, 2008b).

In practice it is not possible to prove that a country or zone is free from infection, instead the aim of this type of surveillance is to provide adequate evidence that infection, if present, affects less than a specified proportion of the population.

Surveillance for determining distribution and occurrence of infection should be designed to collect data about a number of variables, specifically prevalence or incidence of infection, morbidity and mortality rates, frequency of infection risk factors and their quantification, frequency distribution of epidemiological units' sizes, frequency distribution of antibody titres and proportion of immunized animals after a vaccination campaign, amongst other information (OIE, 2008b).

Sentinel programs are useful either to provide evidence of freedom from infection or to provide data on prevalence, incidence and distribution of infection or disease.

2.2.2 Sensitivity and specificity of diagnostic tests

Every diagnostic test, either serological or virological, has two important features which may significantly influence the final result. The sensitivity (Se) and the specificity (Sp) of the test are, respectively, "the proportion of truly positive units that are correctly identified as positive" and "the proportion of truly negative units that are correctly identified as negative" (OIE, 2008b). Ferrari et al. (2005) mentioned in is work Se and Sp values of two different c-ELISA kits for the detection of BTV antibodies. One of the kits presented 99.1% Se and 100% Sp and the other presented the inversed values (Se 100% and Sp 99.1%). Biteau-Coroller et al. (2006) also evaluated Se and Sp of c-ELISA tests for BTV antibodies detection, and face to distinct estimates depending on the number of days post-vaccination (p.v.) and the cut-off point of the test, concluded that the issue of test performance should

become an integral part of the design of disease surveillance and that suitable data should be collected for test evaluation.

Despite of the selected approach in BTV surveillance, the sensitivity and specificity of the diagnostic tests employed are key factors in the interpretation of the results obtained. Ideally, these test features should be "validated for the vaccination/infection history and the different species in the population" (OIE, 2008c), since both are population-specific values that are only estimable using an epidemiological approach (Greiner & Gardner, 2000). Every test-based estimator (prevalence, incidence…) presents systematic errors and its value should be adjusted according to Se and Sp of the test used. A systematic review of available data on these test parameters should be the first adjustment step (Greiner & Gardner, 2000). An effective procedure for following up positive results in order to determine with a higher level of confidence whether they are indicative of infection or not, is another frequent way of avoiding false positive results and consequently another adjustment procedure. Singer, Boyce, Gardner, Johnson and Fisher (1998) concluded that "in areas where BTV is endemic and multiple serotypes of BTV circulate, using both the PCR assay and a serogroup test such as c-ELISA or AGID provides the most information for assessing the epidemiology of BTV in an area".

2.2.3 European legislation on BTV surveillance and monitoring

Commission Regulation (EC) nº 1266/2007 lays down rules for the control, monitoring, surveillance and restrictions on movements of animals in relation to bluetongue.

According to this Regulation, Member States (MS) shall implement monitoring programs in restricted zones and surveillance programs outside restricted zones.

Monitoring programs

Monitoring program shall provide information on the dynamics of bluetongue in a zone already subjected to restrictions. Geographical units of reference shall be defined by a grid of 45 x 45 km, unless specific environment conditions justify a different size, and the program must consist at least of i) serological monitoring with sentinel animals and ii) entomological monitoring. Sentinel animals shall be serologically monitored at least every month during the period of vector activity and their minimum number per geographical unit must be representative and sufficient in order to detect a monthly incidence of seroconversion of 2% with a 95% confidence in each geographical unit. Entomological monitoring shall consist of an active program of vector catching with permanently sited aspiration traps equipped with ultraviolet light. At least one trap must be located in each 2000 km^2geographical unit and

every trap must be operated during the night and operate at a rate of at least one night per week. A proportion of the midges collected in the traps must be sent to a specialized laboratory capable of counting and identifying Culicoides species on a routine basis.

Surveillance programs

Surveillance programmes shall detect virus circulation in a bluetongue free area and must consist at least of i) passive clinical surveillance, ii) serological surveillance and iii) entomological surveillance. Passive clinical surveillance shall include an early warning system of reporting suspicious cases (which must be investigated immediately after report), and must be specially reinforced during the vector activity season. Serological surveillance might be performed through random or targeted serological and virological testing of susceptible animals and sample size must be adequate to detect a prevalence of 0.5% with 95% confidence in the bovine population of an epidemiologically relevant geographical area. Entomological surveillance outside restricted zones shall consist of the same annual programmes of vector catching aimed at gathering information on the proven and potential vector species, their distribution and seasonal profiles in an epidemiologically relevant geographical area (Commission Regulation (EC) nº 1266/2007).

Bluetongue network system

Commission Decision 2007/367/EC granted the OIE BT reference centre - Istituto Zooprofilattico Sperimentale - the financial support for the development and establishing of a web-based system to collect, store and analyze bluetongue surveillance data of all MS. The BlueTongue Network application (BT-Net) is currently available to all MS and its full use is of fundamental importance. Commission Regulation (EC) nº 1266/2007 establishes that MS shall transmit to the BT-Net system information on bluetongue gathered during the monitoring and/or surveillance programmes, specifically i) a monthly report containing data on the serological and entomological monitoring programmes (inside of the restricted zones), ii) an intermediate report covering the first six months of the year, containing at least data on the surveillance programmes (outside of the restricted zones) and on the vaccination inside of restricted zones and iii) an annual report which shall gather the above mentioned information regarding a one year period (Commission Regulation (EC) nº 1266/2007).

2.2.4 Surveillance of BT in Austria

Serological surveillance

Within the framework of the surveillance system for bovine brucellosis, bovine leucosis and bovine herpesvirus, where 3000 holdings were selected according to a risk based sampling plan, the Austrian authorities started to test serologically these samples for BTV-8 as well, in 2007. With the findings of BTV-8 cases in the Netherlands, Belgium and Germany, the Austrian authorities divided the country into 28 sentinel zones (Figure 7) in the beginning of 2008 but still, only the samples from the surveillance system were tested. When outbreaks occurred in the southern part of Germany, in July 2008, the sentinel zones 1 and 2 of Austria fell into the restricted zone and since that time the sentinel herds lying in these zones were tested (Figure 8). That means that in each of these zones, once a month 10 sentinel herds and 10 animals per herd were serologically tested against BTV-8 neutralizing antibodies with c-ELISA. In the remaining 26 sentinel zones only the above mentioned samples from the surveillance system were tested. The results from the surveillance system samples and from the sentinel program were together included in monthly reports of the "Bluetonguevirus Projekt". In November 2008, with the occurrence of the first BT case in Austria within the sentinel zone 25, the Austrian authorities had to implement the sentinel program in 15 other sentinel zones, included in the restricted zone around that first BT case (Figure 8). Since the beginning of 2009 the whole country is under the sentinel program and no surveillance samples are tested anymore.

Figure 7 – Austrian sentinel zones

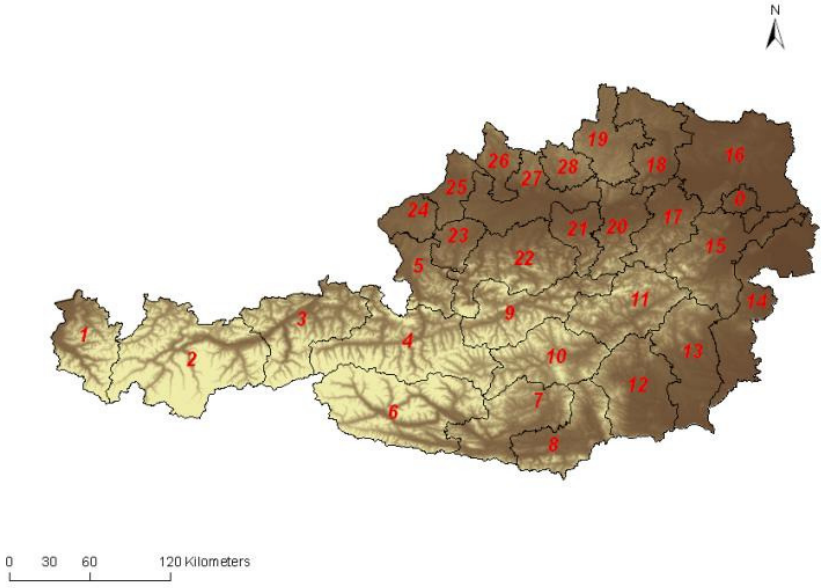


Figure 8 – Sentinel zones included in the restricted zone around the south German BT outbreaks, in July 2008.

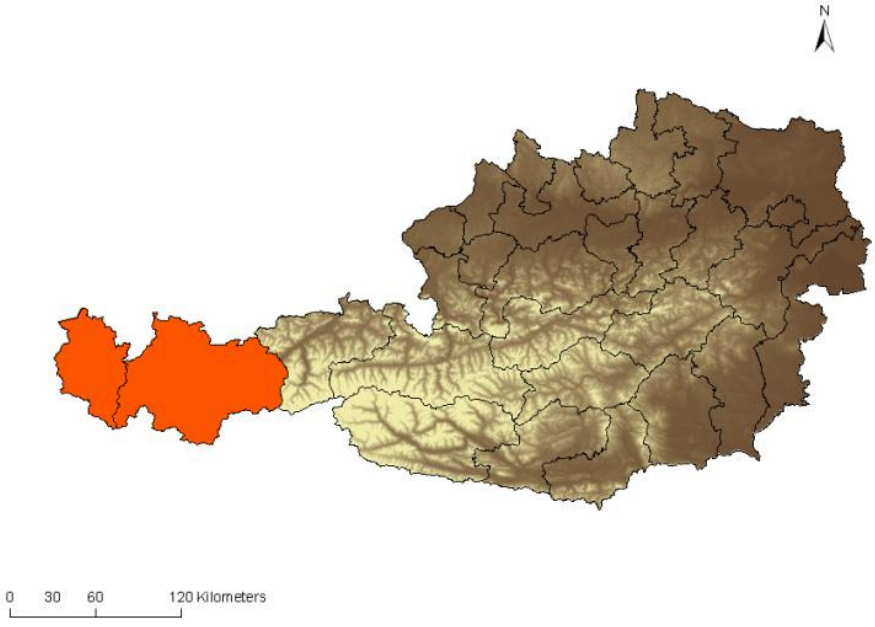
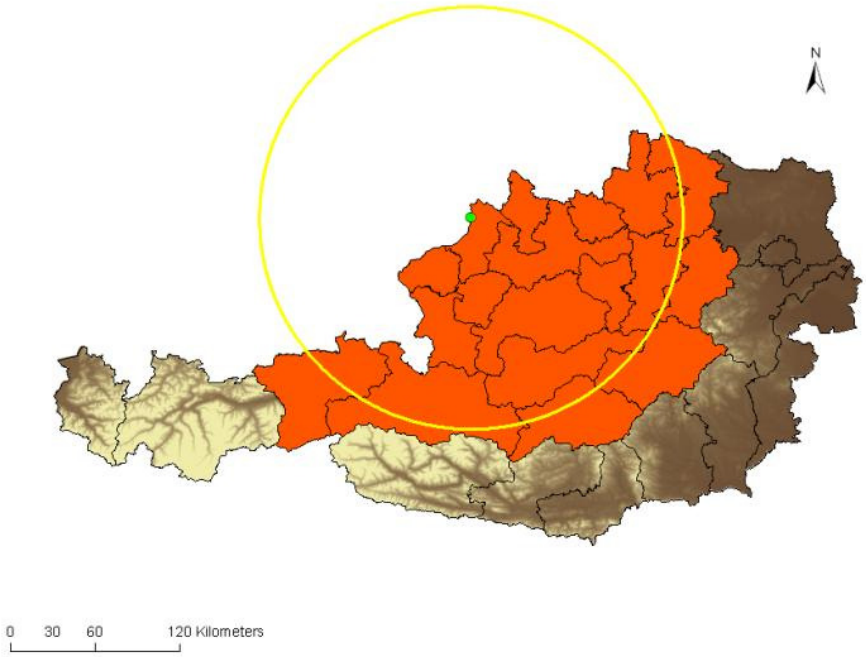


Figure 9 – Sentinel zones included in the restricted zone around the first BT case in Austria, in November 2008.



Vector surveillance

In June 2007, a project of *Culicoides* surveillance in Austria was started between the Bundesministerium für Gesundheit Familie und Jugend (BMGFJ), AGES and the International Research Institute of Entomology (Natural History Museum Vienna). Around fifty blacklight traps have been set up over the whole Austrian territory, distributed at locations chosen according to a 40 x 40 km raster grid, and activated every Monday from dusk until dawn (Sehnal, Schweiger, Schindler, Anderle & Schneemann, 2008; Silbermayr, 2009). Sampling sites were farms with at least 10 ruminants, located near to water bodies and at low sea levels (maximum altitude of 1190 m), and which did not practice transhumance. The traps were set up at weather-proof areas outside of the stable and out of the animals' reach (Sehnal et al., 2008).

In 2007, 87.3% of the collected *Culicoides* were assigned to the Obsoletus complex, 6.7% to the Pulicaris complex and 0.1% to the Nubeculosus complex. Neither *C. imicola* nor *C. dewulfi* were detected in Austrian territory, in that year (Sehnal et al., 2008).

2.3 Control and eradication of Bluetongue

According to Council Directive 2000/75/EC, which lays down specific provisions for the control and eradication of bluetongue, when the presence of the disease is officially confirmed, the country shall implement a group of measures in order to avoid the extension of the epidemic. The slaughter of infected animals and the destruction of their carcasses shall be carried out as well as epidemiological surveys and the prohibition of animal movement from and to infected holdings. In addition, the competent authority shall demarcate a protection zone (PZ) and a surveillance zone (SZ), taking into account geographical, administrative, ecological and epidemiological factors related with BT. The protection zone "shall consist of a part of the Community territory having a radius of at least 100 kmaround the infected holding" (Council Directive 2000/75/EC) and the surveillance zone "shall consist of a part of the Community territory with a depth of at least 50 kilometers extending beyond the limits of the protection zone and in which no vaccination has been carried out during the previous twelve months" (Council Directive 2000/75/EC). The PZ and SZ together constitute the restricted zone (RZ), as established in Commission Regulation (EC) nº 1266/2007. The main BT control measures, specifically vaccination, protection against vectors and restriction on animal and animal products movements, differ between PZ and SZ as well as between RZ and non-RZ.

MS shall ensure that in the PZ i) all holdings with animals inside the zone are identified, ii) an epidemiosurveillance program based on sentinel animals serological monitoring and vectors monitoring is implemented and iii) a ban on the exit of animals from the PZ is applied. Additionally to these measures, the BT vaccination of animals inside of the PZ may be decided (Council Directive 2000/75/EC). In the recently published Commission Regulation (EC) nº 123/2009 it was established that in a PZ where vaccination was carried out, the MS may demarcate a part of that PZ as a "restricted zone with vaccination and without circulation of bluetongue virus of a specific serotype or serotypes" (Commission Regulation (EC) nº 123/2009), also denominated as a "lower-risk area".

2.3.1 Restrictions on movements of animals and animal products

Within the same RZ, movements of animals shall be allowed by the competent authority, provided that the animals to be moved do not show any clinical signs of BT on the day of the transport. However, movements from a PZ to a SZ or to a lower-risk area, or from a RZ to a free-zone require an exemption from the exit ban, which may only be authorized if certain requirements are complied or if the animals are destined for immediate slaughter and it is provided that

> a) no case of bluetongue has been recorded in the holding of origin for a period of at least 30 days prior to the date of dispatch; b) the animals are transported under official supervision directly to the slaughterhouse for slaughter within 24 hours of arrival at the slaughterhouse of destination; c) the competent authority at the place of dispatch notifies the intended movement of the animals to the competent authority of the place of destination at least 48 hours prior to the loading of the animals (Commission Regulation (EC) nº 1266/2007).

The exemption from the exit ban for other purposes than not the immediate slaughter of the animals is only authorized if certain requirements are complied. These requirements are extensively described in Commission Regulation (EC) nº1266/2007, and summarized in the 2008 OIE code as a group of recommendations for the importation of ruminants and other BTV susceptible herbivores from BTV infected countries or zones. The OIE code recommends that the veterinary authorities should require the presentation of a certificate attesting that the animals

> i) were protected from attack from *Culicoides* likely to be competent BTV vectors since birth or for at least 60 days prior to shipment; or ii) were protected from attack from *Culicoides* likely to be competent BTV vectors for at least 28 days prior to shipment, and were subjected during that period to a serological test according to the Terrestrial Manual to detect antibody to the BTV group, with negative results, carried out at least 28 days after introduction into the quarantine station; or iii) were protected from attack from *Culicoides* likely to be competent BTV vectors for at least 14 days prior to shipment, and were subjected during that period to an agent identification test according to the Terrestrial Manual, with negative results, carried out at least 14 days after introduction into the quarantine station; or iv) were vaccinated in accordance with the Terrestrial Manual at least 60 days before shipment, against all serotypes whose presence in the source population has been demonstrated through a surveillance programme (…) and were identified in the accompanying certification as having been vaccinated; or v) are not vaccinated, a surveillance programme (…) has been in place in the source population for a period of 60 days immediately prior to shipment, and no evidence of BTV transmission has been detected (OIE, 2008c)

Additionally to these conditions, the animals should i) have been protected from attack from *Culicoides* during transportation to the place of shipment, or ii) have been vaccinated 60 days prior to shipment or had antibodies against all serotypes whose presence in the zones of transit has been demonstrated (OIE, 2008c).

Similar recommendations, which are summarized in the OIE Code regarding the transport of semen, ova and embryos of BT susceptible species, have been established by Commission Regulation (EC) nº 1266/2007.

2.3.2 Protection against *Culicoides* attack

Protecting susceptible animals from the vectors attack is theoretically an effective way of reducing the risk of infection and therefore a mean of controlling the spread of the virus. This control measure assumes particular relevance during animal movements through infected zones. The OIE Code describes as risk management strategies, the following: i) treating animals with insecticides prior and during transportation, ii) loading, transporting and unloading animals at times of low vector activity, iii) ensuring vehicles do not stop during dawn or dusk neither overnight, unless they have an insect proof netting to protect the animals, iv) darkening the interior of the vehicle, v) surveillance for vectors at common stopping and offloading points and vi) using BTV modeling information to identify low risk ports and transport routes (OIE, 2008c). However, according to Commission Regulation (EC) nº 394/2008, experience has shown that the effectiveness of the measures provided to ensure the protection of animals against attacks by *Culicoides* might be undermined by factors such as vector species, climate conditions and the husbandry of the susceptible ruminants. Therefore, that Regulation establishes transitional provisions that MS of destination may require to be applied on the movement of certain animals which are covered by the exemption from the exit ban, on the basis of a risk assessment taking into account the entomological and epidemiological conditions in which animals are being introduced. The period in which these provisions may be applied was extended to December 31st 2009 by the Commission Regulation (EC) nº 1304/2008.

2.3.3 Vaccination

Council Directive 92/119/EEC introduced general measures for the control of animal diseases and according to it, the prevention of diseases in the EU should be based on non-vaccination policies although it is important to make provision for vaccination where such action is demanded in a serious situation. According to this Directive, vaccination against bluetongue may not be carried out by MS except as a supplement to other control measures when an outbreak of the disease occurs, and always in cooperation with the EC. The decision of introducing vaccination in the disease control program of a MS shall be based on

> i) the concentration of animals of the species concerned in the affected zone, ii) the characteristics and composition of each vaccine used, iii) the procedures for supervision of the distribution, storage and use of vaccines, iv) the species and age of the animals which may or must be vaccinated, v) the areas in which vaccination may or must be carried out and vi) the duration of the vaccination campaign (Council Directive 92/119/EEC).

Though, MS may decide to introduce emergency vaccination taking into account the degree of concentration of the animals in certain regions, the need to protect individual breeds and/or the geographical area in which vaccination is carried out.

BT vaccination is, according to Directive 2000/75/EC, prohibited in the surveillance zone. However, in 2008, Commission Decision 2008/655/EC considered that vaccination is the most efficient measure to fight BT and that mass emergency vaccination is the best option to achieve the objectives of reducing clinical disease and losses as well as containing the spread of the disease, protecting free territories and facilitating safe trade. Emergency vaccination may be defined as a vaccination campaign which is implemented for the first time in a territory after the incursion of a new serotype. Decision 2008/655/EC approved emergency vaccination plans against BT of certain MS and fixed the EC's financial contribution for those plans in 100% of the cost of supply of the vaccine and 50% of the costs incurred in carrying out the vaccination. In 2007 and 2008, the EC contributed financially to emergency vaccination plans of Belgium, Czech Republic, Denmark, Germany, Spain, France, Italy, Luxembourg, Netherlands and Portugal (Commission Decision 2008/655/EC). Having in consideration the outbreaks of BT that occurred in the second semester of 2008, particularly the first occurrences of BTV-8 in Austria and Sweden, the new outbreaks of BTV-8 in Denmark and Spain and the further spread of BTV-1 in France, Spain and Portugal, the EC published the Commission Decision 2009/19/EC approving and establishing the EC's financial support to the new emergency vaccination plans submitted by Austria and Sweden and the amended plans submitted by the remaining countries.

Contradicting Directive 92/119/EEC, which considered vaccination as an exceptional measure to apply only face to an outbreak occurrence, the recently published Commission Regulation (EC) nº 123/2009 states that "vaccination in the absence of virus circulation should not be discouraged and preventive vaccination in restricted zones without virus circulation should not be impeded" (Commission Regulation (EC) nº 123/2009).

2.4 Vaccination against bluetongue

2.4.1 BT control with vaccination

After the incursion of BTV into Europe in 1998, the Mediterranean countries used vaccination in order to "minimize direct economic losses to animal production, reduce virus circulation and allow safe movements of animals from endemic areas" (Savini et al., 2008). In fact, these are the three aims of vaccination against bluetongue referred also in the 2008 OIE manual (OIE, 2008a).

The Italian vaccination strategy towards the BTV incursion into Italy in August 2000, was based on a risk assessment (Giovannini et al., 2004c) which "suggested that the vaccination of all domestic ruminants could reduce both direct losses and virus circulation significantly" (Caporale, Giovannini, Patta, Calistri, Nannini & Santucci, 2004). According to this study, the number of secondary cases decreased to less than 1% of the expected number in the absence of vaccination, when the immunization of at least 80% of the susceptible domestic ruminants was undertaken (Caporale et al., 2004). Caporale et al. (2004) states in his paper on vaccination in the control strategy of bluetongue in Italy that "in regions where more than 80% of the target populations were vaccinated properly, the disease disappeared almost completely and virus circulation was significantly reduced, as documented by the serological surveillance system, after a single vaccination cycle" (Caporale et al., 2004).

These findings led to some of the conclusions of the OIE, specifically that "strategic vaccination of all susceptible animals reduced virus circulation" was an observation which deserved further study (OIE, 2003).

Five years later, in the 2008 OIE Manual chapter dedicated to bluetongue, it is stated that "vaccination to prevent the transmission of BTV may be part of a disease control programme. The level of flock or herd immunity required to prevent transmission will depend on the flock or herd size, composition (…) and density of the susceptible population" (OIE, 2008b).

Ward and Carpenter (1997) simulated the effect of herd immunity and age structure on infection of a cattle herd with BTV and observed that increased herd calving rate and decreased age at first calving contributed for the most part of the increase of the prevalence of infection in the cattle herd. The simulation results also suggested that increased herd density provides more vector breeding sites, which although are not necessary to explain a higher prevalence of infection, undoubtedly would contribute to a higher infection rate.

In 16 January 2008, the EU organized a conference on vaccination strategy against bluetongue which was attended by numerous Member State, European and International authorities' members.

Representatives from the EC, EFSA, OIE and from the Portuguese authority Direcção Geral de Veterinária, among others, composed the group of speakers.

To that date, bluetongue vaccination had been already used in Italy, Spain, France and Portugal as a mean of controlling the outbreaks of bluetongue, with successful results (EC, 2008a). The vaccine against BTV8 was, however, still under development, reason why vaccination had not been carried out yet in the northern Europe outbreaks (EC, 2008a).

The EC established close contacts with vaccine producers to ensure the BTV8 vaccine availability for MS as soon as possible (EC, 2008a), in order to proceed with the EC's political line for bluetongue which was outlined as "mass vaccination with all available vaccines" (EC, 2008b). In the same conference it was also concluded that "emergency mass vaccination is the most efficient strategy, taking into account the current European Union (EU) situation" (EC, 2008b).

However, it was established that, in course of 2009, a follow-up and an evaluation of the mass vaccination strategy counseled by the EC, should be carried out (EC, 2008b).

2.4.2 Vaccination strategies

Vaccines against bluetongue may be used for different strategies, depending on the epidemiological situation of the affected country or zone (EFSA, 2007b) and also on "the types of vaccines available and on the vaccination plan" (EFSA, 2007b).

The main purposes of a vaccination strategy against bluetongue are i) the prevention of clinical disease, ii) limiting the spread of the virus in an area, iii) allowing the movement of susceptible animals between affected and free zones and iv) ultimately eradicating the infection from a region or country (Gerbier et al., 2004; EFSA, 2007b; Savini et al., 2008)

In endemic countries or zones, such as South Africa, where the vectors may be present all year-round and where the virus circulation is continuous (Savini et al., 2008) the goal is to prevent clinical disease and reduce animal losses (Dungu, Potgieter, Von Teichman & Smit, 2004b; Savini et al., 2008), and the vaccination plan usually embraces only sheep, since cattle and other domestic ruminants rarely develop severe clinical signs (Verwoerd & Erasmus, 1994; EFSA, 2007b; Savini et al., 2008).

When the virus reaches a previously non-endemic area, as it happened in some Mediterranean countries, the vaccination is used with the purpose of limiting the extension of the infection, permitting the safe movement of susceptible animals and reducing the virus circulation (Savini et al., 2008). Ultimately, the last purpose might lead to the eradication of the infection. For the purpose of eradication, the vaccination campaign should cover all the susceptible ruminants and be extended to wide areas surrounding the outbreaks. However, eradication

through vaccination can only be achieved under concurrent geo-climatic conditions (EFSA, 2007b; Savini et al., 2008) which prevent vector activity, such as cold winters, geographical barriers, low vector densities and low probability of BTV incursion from neighboring zones (Savini et al., 2008).

Vaccination however is not critical in order to achieve eradication. Greek authorities did not use vaccination against BTV, and applied instead strict measures depending on local epidemiological situations and the country was declared free of BTV in March 2005 (EFSA, 2007b).

Nevertheless, when it is decided to reach eradication through a vaccination plan, it is necessary to have in mind the concept of herd immunity threshold (HIT). Gerbier et al. (2004) affirms that "it is very important to evaluate the level of vaccination coverage that should be attained to eradicate disease, specifically the herd immunity threshold" and that "it has been shown that HIT calculation should be based on the evaluation of the strength of spread of disease measured by the basic reproduction ratio R_0" (Gerbier et al., 2004).

The HIT is defined by Georgette (2009) as "the overall fraction of a population that must be vaccinated (regardless of when these vaccinations occur) to reduce the mean number of secondary infections per infectious individual to less than one (…).

The basic reproduction ratio helps assessing the risk posed by a disease to a susceptible population (Gubbins, Carpenter, Baylis, Wood & Mellor, 2008) and is defined "as the average number of secondary cases arising from the introduction of a single infected individual to an otherwise susceptible population" (Gubbins et al., 2008). Higher vaccination coverage reduces the R_0, therefore reducing the number of infections caused by an outbreak (Georgette, 2009). When the estimation of the HIT is not a possibility an empirical evaluation designated as Charles Nicolle's Law should be followed. It states that the HIT should be 80% or higher (Gerbier et al., 2004).

2.4.3 Vaccines against bluetongue

Currently, there are two types of commercially available vaccines against bluetongue (BTV vaccines), which can be used in the EC approved national disease control programs.Those are the MLVs (or live attenuated vaccines) and the inactivated virus vaccines (EFSA, 2007d; Roy et al., 2009). Other groups of vaccines, like the virus-like particles (VLPs) produced with the recombinant baculoviruses system, and the poxvirus-vectored vaccines, are still under development and none has been licensed to date.

As described in the OIE Manual, BTV vaccines must be validated regarding four important parameters: safety, efficacy, transmissibility and reversion to virulence. The last two parameters only constitute an issue regarding the MLVs.

Safety tests consist on the demonstration of avirulence, which is particularly important for MLVs. The test is valid "if all the vaccinated sheep show evidence of virus replication and do not display signs of disease other than mild transient illness" (OIE, 2008a). Teratogenicity is not addressed in the safety tests for MLVs, because according to the OIE it is implicit that these vaccines are teratogenic and "should not be administered to pregnant sheep during the first half of pregnancy" (OIE, 2008a).

Efficacy tests, similarly to safety tests, must be performed with all BTV vaccines. Vaccinated and unvaccinated sheep should be challenged with the virulent homologous serotype of BTV, preferably passaged only in ruminant animals, since passage in ECE or in cell cultures results in less virulent viral cultures. In these tests, unvaccinated animals (control sheep) should show clinical signs of the disease as well as viraemia contrarily to the vaccinated animals. As a further evidence of infection, sera of all animals should be collected before and after the inoculation of the virulent BTV and checked for the presence of neutralizing antibodies (OIE, 2008a). However, previous studies have shown that the immunization of sheep with MLVs and inactivated vaccines often did not induce neutralizing antibodies although the animals resisted challenge with virulent virus (Pearson & Roy, 1993). This might be one of the reasons why EFSA recommends the postulation of absence of viraemia as the best predictor for efficacy of BTV vaccines (EFSA, 2007c).

The tests for transmissibility and reversion to virulence are rarely, if ever performed, and both are MLVs' only related issues. The determination of transmissibility requires that vaccinated sheep are exposed during viraemia to competent, uninfected *Culicoides*, which are then permited to feed on uninfected unvaccinated animals that are monitored for the presence of BTV or anti-BTV antibodies. Reversion to virulence can only be monitored by comparing the virulence of the vaccine virus with that which had been subject to several sheep-insect replication cycles. Procedures to determine those parameters are difficult to perform in laboratory circumstances.

Live attenuated vaccines or MLVs

Live attenuated vaccines descend of field isolates of the virus which are adapted to growth *in vitro* and are serially passaged a number of times in a tissue culture or in ECE (Murray & Eaton, 1996; OIE, 2008a; Savini et al., 2008). The number of cell passages should be the adequate, since too few passages retain the ability of the virus to cause clinical signs and too

many passages generate viruses which will replicate poorly in the host and therefore fail to elicit a protective immune response (Murray & Eaton, 1996). The "stimulation of a strong antibody response by these vaccines is directly correlated with their ability to replicate in the vaccinated host" (Savini et al., 2008).

MLVs have proven to be capable of eliciting a strong and effective immunity against challenge with virulent homologous virus after the administration of a single inoculation (Murray & Eaton, 1996; EFSA, 2007d). The antibodies may appear before day 10 p.v., and persist for well over a year (OIE, 2008a; Veronesi et al., 2005). In addition to their efficacy, the second advantage of this type of vaccines is their ease of production. "The vaccines are cheap to prepare because a relatively small number of attenuated virus particles is required for each dose (the virus replicates in the vaccinated animal)" (Murray & Eaton, 1996). However, BTV MLVs suffer from some documented potential drawbacks when compared to non-living alternatives (OIE, 2008a).

Murray and Eaton (1996) pointed out as the four main deficiencies of MLVs i) the teratogenic effect of the vaccine virus, ii) the presence of the attenuated virus in the semen of vaccinated bulls and rams, during the post vaccination (p.v.) viraemia iii) the transmission of the attenuated virus by the midge vectors from vaccinated to unvaccinated animals, with the possibility that this release of the virus into the environment could lead to a reversion to virulence, and iv) the possibility that attenuated viruses could recombine with wild type strains and "create viruses that have new genetic, and hence biological, properties" (Murray & Eaton, 1996).

Veronesi et al. (2005) developed a study in order to investigate some of the concerns associated with the use of MLV's. The authors used a group of 32 sheep and the commercially available live attenuated vaccines against BTV-2 and BTV-9, in order to assess the development of viraemia, the possibility of transmission by vectors, the reversion to virulence and the reassortment with wild-type viruses. In this study, all 32 sheep developed p.v. viraemia, with a duration which ranged from 9 to 17 days, and peak titres which varied between 2.75 and 6.25 $\log_{10}$ $TCDI_{50}$/ml (50% tissue culture infective dose per milliliter). According to a previous work of Bonneau et al. (2002), a titre of 2.4 $\log_{10}$ $TCDI_{50}$/ml is sufficient to infect vector midges, contradicting the traditionally considered safe threshold of 10^3 $TCDI_{50}$/ml (OIE, 2008a). These results therefore suggested that the vaccine virus replicates in vaccinated animals to sufficiently high titres to allow infection of midges. Further results of Veronesi et al. (2005) showed that vaccine viruses replicate in infected midges to titres higher than 2.5 $\log_{10}$ $TCDI_{50}$/midge, a minimum value necessary to occur transmission from midge to host. These results strongly support the findings of Ferrari et al.

(2005), whose work demonstrated the active circulation of vaccine BTV-2 among unvaccinated cattle in central Italy.

The active circulation of vaccine viruses in the field, which has been proven possible, poses two main concerns: the reversion to virulence and the reassortment with wild-type viruses.

The replication of the vaccine viruses in the vectors and the occurrence of a number of host-vector cycles make the reversion to virulence a critically possible event (OIE, 2008a; Ferrari et al., 2005). According to the OIE (2008a), an appropriate way to monitor the reversion to virulence is difficult to achieve, however in South Africa "it is accepted that if blood from vaccinated animals during the viraemic stages is serially passaged three times in sheep without reversion to virulence, the chances of reversion in the field will be infinitely small" (OIE, 2008a).

Ferrari et al. (2005) considers reassortment as an unlikely event, due to the constant number of BTV serotypes throughout the last decades. However, Murray and Eaton (1996) cited a personal communication of MacLachlan about studies that had shown the presence of an attenuated BTV-10 gene segment in a field isolate of BTV-13 and Veronesi et al. (2005) mentioned a high frequency of reassortment of genome segments in a Culicoides species experimentally infected with BTV-10 and BTV-17, hence it can be assumed that reassortment between vaccine viruses and field viruses, with a possible reversion to virulence of the vaccine strains is possible (Veronesi et al., 2005).

Veronesi et al. (2005) observed moderate to severe clinical signs of bluetongue, although of short duration (maximum of 6 days), in all 32 sheep, suggesting a possible under-attenuation of the vaccine viruses. However, apart from this study, only mild symptoms (transient fever starting from the fifth day p.v. and mild hyperemia of the oral cavity during the second week) were observed in most experimental studies with MLVs (Savini et al., 2008).

In 2007, Savini et al. (2008) also described the disadvantages of BTV MLVs, based on the experience of the Mediterranean countries with attenuated vaccines against the serotypes 2, 4, 9 and 16 imported from South Africa after the incursion of BTV into Europe in 1998. According to the authors, the explanation of some of the drawbacks of this type of vaccines, in particular the development of clinical signs, lies on an under-attenuation of the field virus during the vaccine production. Among some of the adverse consequences of MLVs are clinical signs of bluetongue including fever, facial oedema and lameness, depressed milk production in lactating sheep, abortions, embryonic deaths and teratogenesis when the females were vaccinated during pregnancy, and a decrease of the semen quality in vaccinated rams. According to these authors, "reports of adverse events in the field greatly vary with the strain of BTV MLV used for vaccination of the animals".

Table 2– Adverse effects associated with different BTV serotype combinations in MLVs.

Vaccine serotypes	Clinical signs	Effect on pregnancy	Effect on semen	Effect on milk production
BTV-2	negligible	< 0.5% abortion in sheep and cattle [c]	decrease of the semen quality; no detection of BTV [d]	negligible
BTV-4	negligible	-	-	-
BTV-16	clinical signs of BT in sheep [a]	-	-	-
BTV-2 & -4	negligible	-	-	negligible
BTV-2 & -9	< 0.1% developed fever and facial oedema	0.53% abortion in sheep and 0.14% in cattle [c]	-	transient decrease of 20 to 30% in production; no changes on milk quality [e]
BTV-2 & -16	-	-	-	transient decrease of 20 to 30% in production; no changes on milk quality [e]
BTV-2, -4 & -9	negligible	-	-	transient decrease of 20 to 30% in production; no changes on milk quality [e]
BTV-2, -4 & -16	clinical signs of BT in sheep [a]	-	-	-
BTV-2, -4, -9 & -16	negligible	-	-	transient decrease of 20 to 30% in production; no changes on milk quality [e]
BTV-1, -2, -4, -9 & -16	significant fever (41-42ºC) [b]	-	-	-

a- monovalent BTV-16 vaccine was discontinued; b- vaccine was never applied in the field; c- BTV detected in < 0.1% of the aborted fetuses; d- results of a study with 23 vaccinated rams; e- effects probably due to the transient perturbation of health induced by the vaccine and not to a direct virus effect on the mammary tissue (Savini et al., 2004).

Table 2 resumes the adverse effects that were associated to each BTV MLV in the cited work. The last disadvantage of MLVs is the impossibility to serologically distinguish naturally infected from MLV vaccinated animals (Savini et al., 2008). The development of a differentiating infected from vaccinated animals (DIVA) strategy is not possible to develop with MLV vaccination.

Considering the description of MLVs and their drawbacks that has been made above, it is not difficult to understand the precautions that are recommended by the OIE on the use of these vaccines: “Attenuated vaccines should be used in the cooler months when the *Culicoides* population and its typical activity are at the lowest level. They should not be used in ewes

during the first half of pregnancy and in rams 2 months before the breeding season" (OIE, 2008a).

Inactivated vaccines

BTV inactivated vaccines are composed by viruses which are produced in large-scale suspension cell systems (proven to be free from contaminating microorganisms), under aseptic and controlled conditions and subsequently inactivated by binary ethylenimine, beta-propiolactone or by gamma irradiaton, purified by chromatography and concentrated by ultrafiltration (OIE, 2008a). The antigens are then made into vaccine by dilution in a buffer solution and addition of adjuvants. "The inactivation process should not significantly alter the immunogenic properties of the viral antigens" (OIE, 2008a), however no live virus should be present at the end of the process. These vaccines are therefore very safe if properly produced (Savini et al. , 2008).

The efficacy of BTV inactivated vaccines has been demonstrated in different studies. Stott, Barber and Osburn (1985) studied the immune response of sheep to inactivated BTV and observed that all inoculated animals developed BTV serogroup-specific nonneutralizing antibodies. Di Emídio et al. (2004) developed a similar study involving sheep, cattle and goats and observed 100% seroconversion following the first injection of an inactivated vaccine in all species. Supporting these findings, the OIE refers that initial studies show that antibodies against BTV can be detected by day 7 p.v. and increase in titre to days 14 to 21 (OIE, 2008a).

According to what is stated in the OIE manual: "data to demonstrate the expected duration of immunity is under development" (OIE, 2008a), therefore the duration of immunity conferred by BTV inactivated vaccines remains still to be established. In fact, the study developed by Di Emídio et al. (2004) showed that at day 137 p.v., all animals that had been immunized with a BTV inactivated vaccine were still protected against challenge, although one year p.v. only goats had neutralizing antibody titres still high. Conversely, a recent study developed by Hamers et al. (2009) with a BTV-2 inactivated vaccine and a group of 7 sheep showed that "a single dose of BTV-2 vaccine given to sheep induces a strong immunity which confers protection for at least one year" (Hamers et al., 2009).

The main advantages of BTV inactivated vaccines are their safety, evident by the absence of systemic reactions related to vaccination, the possibility of developing DIVA strategies (Savini et al., 2008) and the fact that vaccine viruses cannot be transmitted to unvaccinated animals by midges (Murray & Eaton, 1996).

Although this type of vaccine is currently preferred to MLVs, it is not free from disadvantages. Firstly, inactivated vaccines have a high cost of production because, unlike

MLVs, killed BTV does not replicate in the vaccinated animal, therefore the only antigenic stimulus derives from the vaccine itself. For this reason, the administered dose must be significantly high in order to transmit a sufficient amount of antigen (Murray & Eaton, 1996; Savini et al., 2008). Secondly, according to several authors (Murray & Eaton, 1996; Savini et al., 2008; Roy et al., 2009) the vaccination with inactivated vaccines requires two doses (booster immunization) to elicit an immune response, which also contributes for the higher cost of inactivated vaccines, in particular to farmers who have to pay for two veterinary interventions. The need for booster immunizations has been recently contradicted by the previously mentioned study of Hamers et al. (2009). However, Savini et al. (2008) mentioned that a single vaccination did not fully prevent viraemia in animals challenged 7 months p.v. These opposite findings may be due to facts that were already mentioned in the work of Murray and Eaton, in 1996, explicitly the pronounced differences in immunological response to inactivated virus between different breeds of sheep and the different neutralizing antibody responses according to the inactivant used for the vaccine production.

Recombinant vaccines

In 1993, Pearson and Roy wrote about a safer type of BT vaccines that could be engineered through the application of recombinant deoxyribonucleic acid (DNA) technology. With this technique, expression vectors derived from viruses, bacteria, yeasts, animal cells or plant cells can be programmed to synthesize multiprotein structures that mimic virus particles from unrelated agents. Because the multiprotein structures lack the genetic material of the agent, they are not infectious, despite of eliciting protective immune responses against challenge with infectious virus.

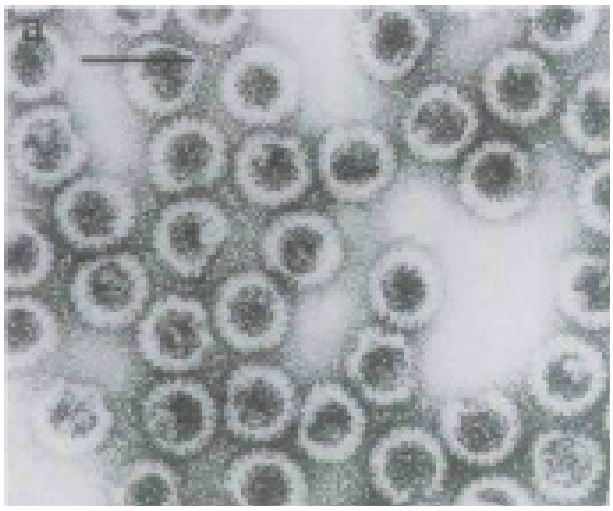
Baculoviruses have been considered as good expression vectors for BTV proteins mainly because they only replicate in particular Lepidoptera species (moths and butterflies), which makes the baculovirus expression system safe to use in the production of vaccines to vertebrate animals, but also because it allows the production of large amounts of immunogens (Pearson & Roy, 1993).

An expression baculovirus is specially developed to express foreign genes. It has non-essential protein encoding genes replaced with foreign DNA (such as cDNA copies of BTV RNA segments), and the promoters that naturally allowed the expression of the non-essential genes will then promote the expression of that DNA. Cultures of Lepidoptera species cells are subsequently infected with the recombinant baculoviruses and synthesize proteins similar in size and antigenicity to those of the foreign infectious agent, such as BTV. The resulting

extracellular virus particles are produced early in infection (from 12 hours onwards) and released by budding from the Lepidoptera cells surface.

By the baculovirus expression system, the resulting multiprotein structures of BTV might be either VLPs or core-like particles (CLPs), depending on the structural proteins that are encoded. The expression of cDNA copies of RNA segments 3 and 7, which encode proteins VP3 and VP7 respectively, results in the production of empty CLPs. According to Pearson and Roy (1993), the CLPs are identical to authentic BTV core particles in size, appearance and stoichiometry (Figure 10).

Figure 10 – Electron micrograph of baculovirus-expressed CLPs.

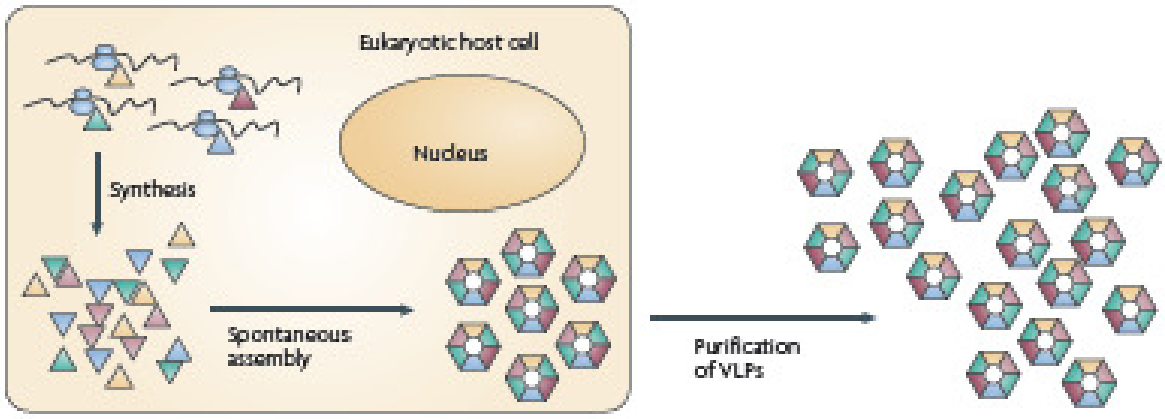


Source of figure: Pearson & Roy, 1993; (a) CLP composed of VP3 and VP7 (x 30 000)

Already in 1993, preliminary vaccination trials have been conducted with CLPs and clinical responses after challenge indicated partial protection against homologous and several heterologous viruses. The CLPs stimulated cell-mediated immunity only and were not able to induce the production of neutralizing antibodies (Pearson & Roy, 1993).The work of Murray and Eaton (1996) supported these findings and attributed the partial-protection observed in CLP-vaccinated animals to the absence of the major serotype-specific protein (VP2) in CLPs. The same work refers that the period of protection conferred by a CLP-vaccination "is unknown but does not exceed 11 weeks after the second vaccination" (Murray & Eaton, 1996).

When Lepidoptera cells are co-infected with two dual recombinant baculoviruses, one encoding VP3 and VP7 genes and the other encoding the outer capsid BTV proteins VP2 and VP5 genes, the co-expression of the four structural proteins leads to the assembly of double-shelled virus particles, with the same size and appearance as authentic BTV virions (Pearson & Roy, 1993). These particles are designated VLPs (Figure 11 and Figure 12).

Figure 11 – VLPs synthesis in Lepidoptera cells infected with two dual recombinant baculoviruses.



Source of figure: Roy et al. (2009); The structural proteins of BTV co-assemble without the need for active virus replication.

Figure 12 – Electron micrograph of baculovirus-expressed VLPs.

Source of figure: Pearson & Roy, 1993; (b) double-shelled VLP with VP2 and VP5 attached to VP3 and VP7 (x 30 000).

Vaccination trials have been also conducted with VLPs. Pearson and Roy (1993) showed that unlike to what was observed with CLP-vaccination, the VLP-vaccinated animals responded to challenge with neutralizing antibodies, although the titres were related to the adjuvant used in the vaccine. Administering VLP without an adjuvant resulted in no response or response to challenge with a low titre of antibodies, whereas the combination of VLP with different adjuvants elicited antibody responses in varying titres (Pearson & Roy, 1993).

After vaccination with two separate doses of a VLP vaccine with a 28 day interval, the duration of immunity was analyzed. Pearson and Roy (1993) observed that sheep maintained almost constant levels of antibodies for more than one year and that sheep immunized with mixtures of BTV-10 and BTV-17 VLPs responded to challenge against both serotypes and also to the related serotype BTV-4, but not to the unrelated BTV-11.

Summarizing, according to Pearson and Roy (1993), the attractiveness of the baculovirus recombinant system lies on the following factors: i) only 10 μg VLP can induce protective and lasting B cell and T cell response in vertebrate hosts; ii) large quantities of VLP can be produced due to the expression capabilities of baculovirus vectors (…); iii) CLP and VLP can be purified using a one-step generic protocol based on the physical properties of the particle (…); iv) they are devoid of any amount of insect, or baculovirus proteins, RNA or DNA; v) the purification procedure is gentle enough to maintain the morphological structure of the particles in their naïve conformations; vi) the particles cannot replicate although they can efficiently attach to cells and be ingested and vii) much information exists concerning the structures. The same authors stated that VLP and CLP vaccines can be as effective but much safer than MLVs or inactivated vaccines. In line with this statement, Murray and Eaton (1996) also assumed that because VLPs contain no viral genetic material, the particles will neither replicate in vaccinated animals nor be transmitted by vector midges to unvaccinated hosts. Nevertheless, VLPs are not free from drawbacks. The production to an industrial level remains an issue to be solved (Murray & Eaton, 1996; Roy et al., 2009) specially concerning to a purification method that could be applied commercially since if not adequately purified, the particles can be contaminated with other insect viruses. Another issue of concern is the fact that to date VLPs have only been tested in the two-dose per animal format (Roy et al., 2009).

A second alternative associated to recombinant DNA technology is the poxvirus-based system, which uses poxviruses "as a vehicle for the transfer of BTV genes into sheep cells, where BTV proteins are then synthesized" (Roy et al., 2009). The vaccinia, capripox and canarypox viruses have all been already used as vectors for the vaccination against BTV with different structural virus proteins. Among those, the canarypox virus is considered a stable and safe vector since it does not replicate in mammals. Savini et al. (2008) mentioned a vaccinia virus that expressed both VP2 and VP5, as well as a capripox virus expressing VP7, both showing reasonable results. However development of both vaccines was not pursued forward. More recently a recombinant canarypox virus VP2/VP5 vaccine has shown to induce highly effective protective immunity in sheep (Savini et al., 2008). There is however one important drawback of poxvirus-vectored vaccines when compared to VLPs. Although the expression of a small fraction of the BTV genes have no risk of acting as a source of virulent BTV, it remains theoretically possible to occur recombination with field strains (Roy et al., 2009).

2.4.4 Vaccination and surveillance

As previously stated, antibodies to BTV may be detected in a serological assay, not only due to infection but also due to vaccination. It is therefore of paramount importance to develop a DIVA strategy for surveillance and trade purposes. It is not only important to differentiate natural infected from vaccinated animals but also to identify vaccinated animals that may have become infected (Barros et al., 2009). Currently, the differentiation between field and vaccine strains of BTV circulating in Europe is only performed by RT-PCR (EFSA, 2007d).

DIVA serological strategies cannot be applied to populations where MLVs were used (Hamblin, 2004; EFSA, 2007d; Bhanuprakash, Indrani, Hosamani, Balamurugan & Singh, 2009) because there is no difference between the immune response after infection with field virus to that occurring after vaccination with MLVs. However, inactivated vaccines have been gradually substituting MLVs in the control of BTV in European countries, and some studies are in progress in order to develop serological tests with DIVA function that can be applied to animals vaccinated with this type of vaccines. Barros et al. (2009) developed an indirect ELISA (i-ELISA) using the recombinant BTV NS3 protein as an antigen and applied this serological test to seven bovines which have been vaccinated with a bivalent inactivated vaccine (against BTV-2 and -4). The NS3 protein is produced in large quantities in infected cells and is essential for both virus assembly and release. A NS3-antibody response is therefore associated to *in vivo* virus activity. Since there is no replication of inactivated viruses in animals vaccinated with inactivated vaccines it can be supposed that NS3-antibodies should not be present in those animal sera (EFSA, 2007d). Furthermore, BTV inactivated vaccines mainly contain capsid proteins, which should make antibodies against BTV non-structural proteins difficult to detect in vaccinated animals. In fact, Barros et al. (2009) observed that none of the immunized animals developed significant NS3-antibody levels, neither after vaccination nor after challenge, although they produced VP7-antibodies that were detected by the conventional c-ELISA. It was therefore concluded that the NS3-based i-ELISA, although less sensitive than RT-rPCR in detecting earlier infections, is an easier and cheaper method with DIVA function for use in routine diagnosis (Barros et al., 2009).

Recombinant vaccines that are presently under development are also suitable for DIVA serological strategies. The lack of expression of some viral proteins in VLPs, CLPs and virus-vectored vaccines allows for the development of appropriate ELISA markers that could be used to distinguish between vaccinated and naturally infected animals (Roy et al., 2009). OIE recommended the development and validation of technologies to distinguish vaccinated from

infected animals, both for current vaccines and those that are likely to be available in the foreseeable future (OIE, 2003).

In summary, an ideal vaccine for bluetongue would protect against as many virus serotypes as possible but would not revert to virulence, and would not recombine with circulating strains of the virus (…), would pose no danger of replicating within insects and would be compatible with tests to distinguish between infected and vaccinated animals (Roy et al., 2009).

2.4.5 Overview of vaccination against BT in Europe

Council Directive 2001/82/EC states that

> In the event of serious disease epidemic, Member States may provisionally allow the use of immunological veterinary medicinal products without an authorization for placing on the market, in the absence of a suitable medicinal product and after informing the Commission of the detailed conditions of use (Council Directive 2001/82/EC).

After the incursion of BTV into Europe, several EU countries used emergency vaccination by implementing this Directive. As no other BT vaccines were available by that time in Europe, MLVs imported from South Africa were used. The use of inactivated vaccines, due to its advantages previously described in this work, was however preferred to the use of MLVs. Therefore, once the inactivated vaccines able to protect against the circulating serotypes became available, they gradually substituted the South African imported vaccines. The inactivated vaccines were produced and controlled in EU and a dossier, even not complete, was provided allowing the assessment of the quality, safety and efficacy of the products. A risk/benefit analysis considered their use favorable even without marketing authorization. However, there is an unequivocal preference to have access to vaccines with a marketing authorization. Within this context, the availability of authorized BT vaccines could be facilitated by a similar approach to the one that was applied for Avian Influenza vaccines during that disease epidemic, under the request of the EC. The Committee for Medicinal Products for Veterinary Use established minimum data requirements for an authorization under exceptional circumstances for inactivated vaccines for emergency use, and the European Medicines Agency adopted an accelerated timeframe for the authorization of vaccines in order to respond to the then prevailing epidemiological situation. So far no request has been made by the EC to set the stage for a similar approach regarding BT vaccines, although this approach can be repeated for BT should the situation require it (EFSA, 2007b).

Savini et al. (2008) described the use of MLVs and inactivated vaccines in European countries since the beginning of BT outbreaks in Europe until 2006.

Table 3 illustrates the use of BT vaccines in domestic ruminants in European countries affected by BTV until 2006.

Table 3 – Bluetongue vaccination campaigns in Europe until 2006.

Vaccine	**Year of vaccination**							
	France	*Italy*		*Spain*		*Portugal*		*Bulgaria*
	Sheep	Sheep	Cattle	Sheep	Cattle	Sheep	Cattle	Sheep
MLV								
BTV-3,-8,-9,-10 & 11	-	-	-	-	-	-	-	1999-2000
BTV-2	2000-02	2002-06	2002-06	2000-01[a]	-	-	-	-
BTV-4	-	-	-	2004-06	-	2005-06	-	-
BTV-2 & 4	2003-04	2004-06	2004-06	2003[a]	-	-	-	-
BTV-2 & 9	-	2002-06	2002-06	-	-	-	-	-
BTV-16	2004	-	-	-	-	-	-	-
BTV-2,-4 & 16	-	2004	2004	-	-	-	-	-
BTV-2,-4,-9 & 16	-	2004	2004	-	-	-	-	-
BTV-2,-4 & 9	-	2005-06	2005-06	-	-	-	-	-
BTV-9	-	-	-	-	-	-	-	-
Inactivated								
BTV-2	2005	-	-	-	-	-	-	-
BTV-4	-	-	-	2005-06	2005-06	2005-06	2005-06	-
BTV-2 & 4	2006	2005-06	-	-	-	-	-	-

Adapted from Savini et al. (2008); a - Balearic Island.

Several monovalent MLVs and MLVs combinations have been used previously to year 2005, when the first inactivated vaccines became available for use against BTV serotypes affecting Europe by then. In 2002, evidence was found of a reassortment between BTV-2 and BTV-16 MLVs, giving rise to a BTV-16 new circulating strain in Italy (Roy et al., 2009). After 2006, MLVs have gradually been replaced by inactivated vaccines in vaccination campaigns against all BTV serotypes circulating in Europe.

2.4.6 Vaccination against BT in Austria

When BTV-8 outbreaks occurred in the southern part of Germany close to the border with Austria, in July 2008, a part of Austria's territory fell into the restricted zone related to those outbreaks. Therefore, since August 2008 the regions of Vorarlberg and Tirol initiated a vaccination programme against that serotype. In November 2008, with the occurrence of the first BT case in the region of Upper Austria, the vaccination programme was extended to that

region and to the northern part of Salzburg, which also fell into the restriction zone. In December 2008 the Austrian Veterinary Authorities decided to extend the vaccination campaign to the totality of the territory (BMGFJ, 2008). All cattle older than 3 months and all sheep and goats older than 4 weeks of age (excepting fattening bulls and oxen, bulls for semen collection and zoo-animals) were included in the programme. There was no differentiation in the vaccination procedures between high- and low- risk zones for BTV-8 occurrence (BMGFJ, 2009). The vaccination campaign was performed with an inactivated vaccine and ended in April 2009. So far there is no available information about a further revaccination plan.

2.5 Research and evaluation of the risk of BTV in Europe

Following the incursion of BTV into Europe in 1998, several works have been published developing models for particular steps of the RA process.

Risk is defined by the OIE as "the likelihood of the occurrence and the likely magnitude of the biological and economic consequences of an adverse event to animal or human health in a specified time period" (OIE, 2008d).

Although some form of risk analysis has always been undertaken in veterinary epidemiology, it is only since the 1990s that documented methodologies have been developed. The components of risk analysis according to the OIE are hazard identification, risk assessment, risk management and risk communication.

Risk assessment (RA) is the component of the analysis which estimates the risks associated with a hazard (an agent or condition of an animal or animal product with the potential to cause an adverse health effect). RA consists of four interrelated steps: release assessment, exposure assessment, consequence assessment and risk estimation. The release assessment describes the probability of the introduction of the potential hazard in a population under each specified set of conditions and how these might change as a result of various actions, events or measures. If it demonstrates a significant risk, an exposure assessment should follow. The probability of exposure to the identified hazard is estimated for specified exposure conditions. If the exposure assessment demonstrates significant risk, the risk assessment should continue further with the consequence assessment. This step describes the potential consequences of a given exposure and estimates the probability of them occurring. Finally, risk estimation consists of integrating the results from the release assessment, exposure assessment, and consequence assessment to produce overall measures of risk.

RA may be qualitative or quantitative. Qualitative assessment does not require mathematical modeling skills whereas quantitative assessment is represented by mathematical models

where the inputs and outputs are expressed numerically. In the past, risk assessment in veterinary medicine has been performed qualitatively. However, with the establishment of the World Trade Organization and the promulgation of the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement), quantitative methods have become increasingly important, especially in their application to international trade of animal and animal products (Giovannini et al., 2004b). According to De Jong (1995), mathematical modeling is advantageous for the study of population dynamics of infectious agents, since models show how separate measures can be seen as a manifestation of the same underlying process and because the main factors affecting infection rates are not additive but multiplicative. Similarly, Wilesmith (1997) defended that "one of the uses of applying mathematical modeling is to identify key aspects to which the dynamic processes are most sensitive" and that "this provides important pointers as to where efforts should be directed". The simplest form of a quantitative RA is a deterministic or point estimate analysis, where both the inputs and outputs are expressed as single numbers or point values. For more complex models, probabilistic or stochastic analyses are preferable. In these, inputs are described as probability distributions (OIE, 2004).

The SPS agreement recognizes the OIE as the international organization responsible for the development of international health standards, guidelines and recommendations affecting animal trade. The OIE provides recommendations and principles for conducting transparent, objective and defensible qualitative and quantitative RA for animal trade (OIE, 2008f).

According to the OIE, the main principles of RA are: i) it should be flexible to deal with the complexity of real life situations and no single method is applicable in all cases; ii) both qualitative and quantitative RA methods are valid; iii) the assessment should be well-documented and supported with references to the scientific literature and other sources, including expert opinion; iv) consistency and transparency in RA methods are essential; v) RA should document the uncertainties, the assumptions made, and the effect of these on the final risk estimate and vi) the RA should be amenable to updating when additional information becomes available (OIE, 2008e).

Several RA were made in Europe. Quantitative release assessments have been developed to assess the risk of introduction of BTV through animal movement. Giovannini et al. (2004b) observed that when more than 80% of the susceptible population was vaccinated in the area of origin, the risk associated with the animal movement from restricted areas to free areas appears acceptable. Hoar, Carpenter, Singer, and Gardner (2004) concluded that BTV introduction into a new area through animal movement depends on the number of viraemic animals that enter the area and the competency of local vectors to transmit the virus. Recently,

transmission models have been developed for the BT infection, through the computing of R_0 numbers. R_0 is defined as the expected number of secondary infections arising from a single individual during its infectious period, in a population of susceptibles. It serves as a threshold parameter that predicts whether an infection will spread (Heffernan, Smith & Wahl, 2005), therefore it can be used as a final step of an exposure assessment. It is accepted that only if R_0 is higher than 1, a disease is able to invade a host population (Gubbins et al., 2008) and that once it has "become less than unity, the outbreak will eventually cease regardless of its exact value within the range from 0 to 1" (Georgette, 2009). Gubbins et al. (2008) developed a stochastic model for the R_0 to assess the risk of BT to United Kingdom livestock. Racloz, Venter, Griot and Stärk (2008) developed a similar but deterministic model for R_0 to estimate the temporal and spatial risk of BT in Switzerland.

Risk assessment is a process for which many other different skills are needed. While epidemiology and statistics skills are needed to perform quantitative models, skills on geographical information systems (GIS) are necessary to produce spatial distribution maps that could help revealing any underlying geographical patterns of exposure, disease or infection. It is accepted that exposure as well as disease or infection are not uniformly distributed geographically and that risk patterns usually have both spatial and temporal components (Järup, 2000). Classic epidemiological analysis focused mainly on the animal pattern of diseases, whereas time and space dimensions were poorly explored. GIS has allowed for the analysis of these two dimensions and has therefore become an indispensable tool in epidemiological analysis of animal diseases.

Incorporating GIS into national disease surveillance systems will allow for the improvement of disease surveillance and control methods based on higher spatial resolution (Pfeiffer, 2008; Racloz et al., 2008).

Disease mapping can help to identify possible disease clusters, to define and monitor epidemics and to show changing patterns of health over time. Furthermore, when combined to exposure mapping, it may be useful in exploring causal relationships with exposure. The same is true for infection mapping. However, special caution should exist when interpreting apparent associations between exposure and disease or infection; the modifiable area unit problem (the boundaries of the areas to be studied are often administrative and may not necessarily be ideal for mapping disease or infection) and the latency or incubation period (whether it respects to disease or infection, respectively) should always be taken in consideration.

Furthermore, GIS can be used to estimate the number of expected cases of a disease or infection by combining exposure maps with demographic data of population at risk, as far as

the exposure-response relationships are well known. However, considerable margins of uncertainty are likely to occur around these estimates, when risk is calculated based upon simple mapping and overlaying techniques. Statistical modeling is therefore a more formal technique of assessing the expected number of disease or infection cases (Järup, 2000).

GIS are also very useful in assessing the occurrence of vector-borne diseases, in areas where the landscape elements critical to vector survival are known and can be detected with remote sensing techniques.

After the BTV emergence in Europe in 1998, several studies have been developed in order to map the BT disease and the *Culicoides* distribution and to find geographical correlations. Tatem et al. (2003) has shown that the presence of *C. imicola* in the Mediterranean region was strongly correlated with middle infra-red reflectance, and consequently also correlated with vegetation structure and surface temperature, and how much each varied throughout the year. Caligiuri et al. (2004) implemented a GIS to study areas of the Campania region in Italy that could be potentially at risk for BT infection based on the vectors presence. Risk maps for the presence of *Culicoides* were built based on environmental, meteorological and climatic features. Conte et al. (2007) performed a comparative mapping of the 100 largest collections of *C. imicola* and of the Obsoletus complex in Italy and found them to be strongly divided due to certain biotic and abiotic factors that either promoted or suppressed each species proliferation. The authors' findings indicated that *C. imicola* and the Obsoletus complex do not share a common habitat. Silbermayr (2009) created risk maps of BT occurrence in Austria, for spring, summer and fall seasons, by combining risk maps of *Culicoides* distribution and data of cattle density. Racloz et al. (2008) combined GIS mapping with statistical modeling, specifically risk maps for *Culicoides* presence with maps highlighting different vector activity levels based on R_0 values for BT obtained by statistical modeling.

Chapter3: Analysis of 2008 BT cases in Schärding

An analysis of the cases of the first BT outbreak in Austria was undertaken, using the active surveillance data to estimate the most likely time period when infections could have occurred and to perform a spatial analysis of the cases.

3.1 Material and methods

3.1.1 Data sources

For the analysis of the BT cases occurred in the district of Schärding in 2008, the following data sources were provided by AGES: i) a dataset describing the samples tested (serologically and virologically) for the presence of BTV-8 in Austria, under the active surveillance program; ii) November and December 2008 monthly reports from the "Bluetonguevirus Projekt" of the Austrian Ministry for Health, Family and Youth containing the results from the above mentioned surveillance program; iii) a dataset with average numbers of cattle, sheep and wild ruminants per district in Austria; iv) maps of the average monthly temperatures recorded in 2008 in each capital of region in Austria; v) shapes of the Austrian territory (districts and parishes) and of the geographic location of the BT cases reported in year 2008 in Austria.

Additionally, the reports on bluetongue situation submitted to the Standing Committee on the Food Chain and Animal Health (SCFCAH) by the Austrian Ministry for Health, Family and Youth (BMGFJ, 2008; BMGFJ, 2009) and by the Federal Ministry of Food, Agriculture and Consumer Protection of the Federal Republic of Germany (Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz [BMELV], 2008) also contributed with information for the analysis.

3.1.2 Software tools and analytical methods

The data management and processing were made using the software packages of Microsoft Office Excel 2003 and Access 2003. The built map was made with the use of gvSIG 1.1.9.

The approach of this chapter included the analysis of active surveillance data from Schärding, the investigation of the most likely infection moment of each positive animal found and the examination of the spatial distribution of BT cases. The results were further compared to the EFSA's conclusions on the BTV-8 epidemic in northern Europe in 2006. According to Commission Regulation (EC) nº 1266/2007 one of the possible definitions of "case of bluetongue" is "an animal which has tested positive to bluetongue serological tests or from

which viral antigen or viral ribonucleic acid (RNA) specific to one or more of the bluetongue serotypes has been identified" (Commission Regulation (EC) nº 1266/2007).

Available data on sero-diagnosis of BTV-8 included serum surveillance, pre-movement tests and privately required tests results. In this study, only the serum surveillance results were considered as the other two sub-samples could not be assumed as representative of Schärding's cattle population. Austrian authorities required that every sera positive or suspicious to c-ELISA was further retested with RT-rPCR tests (A. Loitsch, personal communication, May 4, 2009). It is important to have in consideration that only the positive results were further confirmed to avoid the occurrence of false-positives; however no measures were taken in order to avoid false-negatives. The number of infected cattle may be, for this reason, under-estimated.

In conclusion, the case definition adopted is an animal from the species *Bos taurus* (domestic cattle) from which bluetongue serotype 8 specific RNA has been identified by RT-PCR, after a previous positive result with c-ELISA, independently of the observation of clinical signs.

3.2 Results and discussion

Analysis of active surveillance data

In the two-month period from beginning November to end of December 2008, 463 cattle sera samples from Schärding were tested with c-ELISA. Twenty nine of those samples were positive, but after further retesting with RT-PCR, only 10 were confirmed as BT cases. The description of those cases is shown in Table 4.

Table 4 – Description of BT cases occurred in Schärding in late 2008.

Case	Date of serum collection	Species	Gender	Age[a]	Clinical signs
1	11/06/2008	Cattle	F	11	No
2	11/18/2008	Cattle	NA	5	No
3	11/18/2008	Cattle	NA	15	No
4	12/02/2008	Cattle	F	4	No
5	12/02/2008	Cattle	F	3	No
6	12/03/2008	Cattle	F	1	No
7	12/17/2008	Cattle	F	7	No
8	12/17/2008	Cattle	M	1	No
9	12/17/2008	Cattle	M	1	No
10	12/17/2008	Cattle	F	5	No

a – in years; M- male; F- female; NA- not attributed

EFSA's findings (EFSA, 2007a) have conducted to the idea that BTV spread within cattle herds might be more significant than within sheep herds, due to higher duration of viraemia in cattle. Additionally, as described by Koumbati et al. (1999), cattle are more attractive to *Culicoides* than sheep. The 7 farms where BT cases were identified included 3 cattle-only farms and two mixed farms (with cattle, sheep and poultry); no data was available on the population of the two remaining herds. Cattle population in those farms varied between 12 individuals (in a mixed farm) to 32 (in a cattle-only farm). Although herds in Schärding are not high in number (only approximately 5% of the farms have more than 100 cattle), there are around 1400 farms with cattle in this district. This fact may have contributed for the spread of BTV-8 in Schärding after the first case occurrence.

According to EFSA report, wild ruminants may also play a potential role in the spread of BTV-8 (EFSA, 2007a). These animals are present in the district of Schärding, in numbers around 500 animals (fenced wild ruminants), according to AGES data.

Moment of infection

c-ELISA assays have proven to first detect anti-BTV specific antibodies in infected animals at day 7 p.i. (Koumbati et al., 1999; Zhou et al., 2001; Batten et al., 2008). The onset of viraemia starts earlier. BTV RNA becomes detectable by RT-PCR between days 2 and 5 p.i. and remains detectable as far as 200 days p.i. in infected cattle (Bonneau et al., 2002).

Table 5 – Expected periods of infection of the BT cases detected in Schärding.

Case	Date of serum collection	Expected period of infection
1	11/06/2008	4/20/2008 - 10/30/2008
2	11/18/2008	5/02/2008 - 11/11/2008
3	11/18/2008	5/02/2008 - 11/11/2008
4	12/02/2008	5/16/2008 - 11/25/2008
5	12/02/2008	5/16/2008 - 11/25/2008
6	12/03/2008	5/17/2008 - 11/26/2008
7	12/17/2008	5/31/2008 - 12/10/2008
8	12/17/2008	5/31/2008 - 12/10/2008
9	12/17/2008	5/31/2008 - 12/10/2008
10	12/17/2008	5/31/2008 - 12/10/2008

The samples previously described in Table 4 correspond to animals that had been infected in a certain moment in the past, between 200 and 7 days earlier to the date of sample collection, since the diagnosis of BTV infection was first performed with c-ELISA followed by a

confirmation with RT-PCR. Table 5 resumes the expected time interval when infection of each animal most likely occurred.

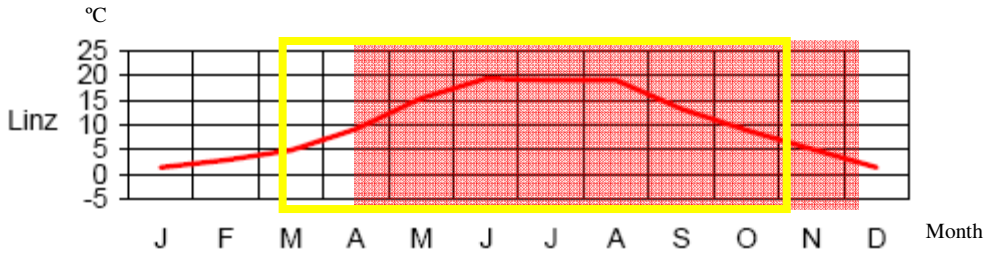
According to these results, BT cases found in Schärding descended from infections that could have occurred between April 2008 and December 2008. Since the case reporting derives from active surveillance and therefore do not match the moment of infection, it was not possible to draw a temporal pattern of the outbreak.

Amongst the factors that EFSA enumerated as useful to predict virus persistence, temperature was the one found to be correlated with the variation of the number of vectors, and a lag of 4 weeks between a change in temperature and a change in the reported number of cases was estimated. Silbermayr (2009) analyzed *Culicoides* catches in Austria in the year 2008 and concluded that the optimal climatic conditions for the vector presence "were identified as being between 10-20 ºC mean daily temperature and between 65-80% relative humidity" (Silbermayr, 2009).

Data on monthly average temperatures was only available for regional capitals. Linz is the capital of Upper Austria, the region where Schärding is located.

Figure 13 illustrates the average monthly temperatures in Linz in the year 2008.

Figure 13 – Average monthly temperatures in Linz, in 2008.



Adapted from AGES. The yellow border delimitates the period of four weeks earlier to the hypothetical dates of infection, in which high *Culicoides* abundance presumably were observed; the period composed by the hypothetical dates of infection is showed in red.

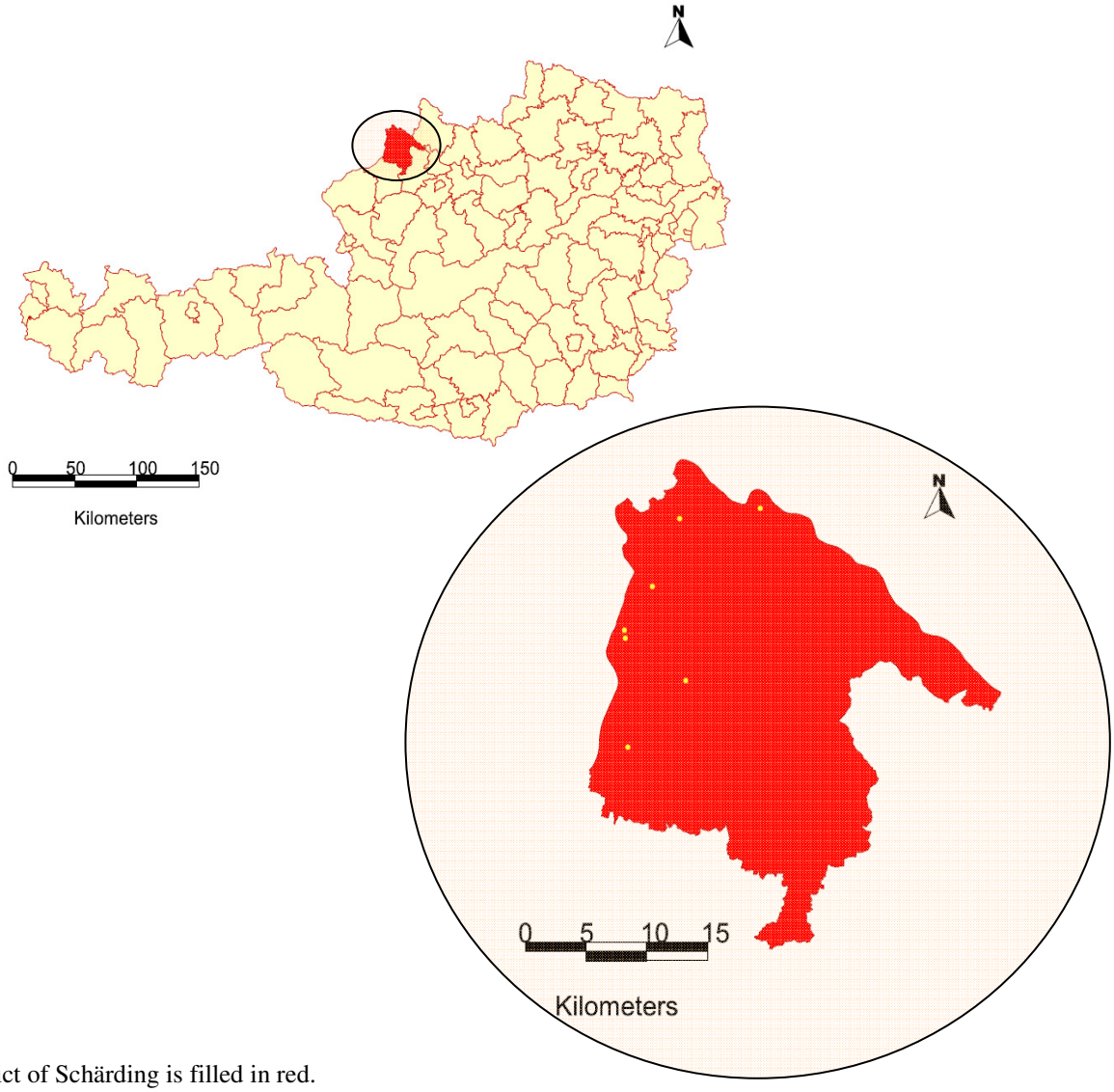
According to the above explained assumption, the moments of infection most likely occurred four weeks after time periods with average temperatures between 10 and 20 ºC. Figure 13 shows that these temperatures were verified inside of the expected period of high *Culicoides* abundance, between middle April to late September, therefore, the detected BTV cases most likely corresponded to infection moments between middle May and late October.

Spatial distribution

The spatial location of the ten cases found in Schärding between November and December 2008 is illustrated in Figure 13. The cases took place in seven different farms (one of the

farms registered two cases and another one three), distributed by five different parishes (Brunnenthal, Esternberg, St. Marienkirchen, Wernstein-Inn and Freinberg), all included in the same sentinel zone.

Figure 14 – Geographic location of the seven farms affected by BTV-8 in the district of Schärding in 2008.



District of Schärding is filled in red.

During the 2006 BTV-8 epidemic in northern Europe, the observed speed of local spread was about 2 km per day, approximately 15 km per week. The maximum distance between affected parishes in Schärding was measured around 21 km (from Esternberg to St. Marienkirchen), but the time interval between the moment of infection of each case is unknown as these moments could not be determined.

BTV-8 cases in northern Europe epidemic showed an upper altitude limit of approximately 650 m (EFSA, 2007a) although *Culicoides obsoletus* abundance had been already recorded in

regions located up to 1211 m (Silbermayr, 2009). The district of Schärding has an altitude of 316 m (Sehnal et al., 2008), which makes it a suitable zone for the establishment of *Culicoides obsoletus* and for BT occurrence, as far as altitude is concerned. In fact, the results of the vector surveillance system set up in late 2007 and across the year 2008, which included one blacklight trap in the district of Schärding, have shown that the predominant majority of *Culicoides* spp. found in Austria belonged to the Obsoletus complex (more than 80%), followed by *Culicoides pulicaris* (more than 5%) (Sehnal et al., 2008; Silbermayr, 2009).

The district of Schärding is situated close to the border with the south eastern region of Germany, Bavaria, where there were occurrences of BTV-8 prior to the start of the outbreak in Schärding. This district was enclosed by both zones of protection and surveillance delineated around the BTV-8 cases occurred in Bavaria prior to November 2008. According to EFSA's report, BTV-8 in nortern Europe tended to spread in an east-west and southward direction, most likely due to the spread of vectors by the wind. It is therefore possible that the outbreak in Schärding was related to the previous occurrences in Bavaria. However, wind analysis was not performed and all the possibilities of virus introduction must be considered, amongst them the import of infected ruminants or live ruminant products, introduction of infected vectors through import of animals, plants or other products or transhumance of infected wild ruminants.

Chapter 4: Comparison of the two districts affected by BTV-8 in Austria in 2008

BTV-8 was identified in late 2008 in two different Austrian districts, Schärding and Bregenz. The initial goal of this chapter was to perform a case-control study involving these two epidemiologically equivalent districts to assess the effect of mass vaccination, which was preventively performed in Bregenz and not in Schärding.

However, data constraints did not allow for that analysis performance. The accessible data from Schärding resulted from an active surveillance program, specifically from the serological testing of sentinel cattle respecting the period between November and December 2008. However, the only available data from Bregenz was the result of a passive surveillance system, specifically from the serological testing of a limited number of s cattle suspected to be infected after the identification of the first BT case in that district, in December 2008.

In order to perform the initially intended analysis, it would be necessary to have access to comparable data from both districts. More specifically, data should be resulting from the same type of surveillance strategy, preferably active surveillance, should consider the same time period and samples should both be representative of the target population in each district.

Still, a hypothesis testing was performed with the available data, with the goal of developing the methodology. The only possible analysis to perform was to test the hypothesis of the existence of a statistical association between the district of origin and the proportion of c-ELISA positive samples which further resulted RT-PCR positive.

4.1 Material and methods

4.1.1 Data sources

In order to develop the present chapter, the data sources mentioned in chapter 1 were used together with the results of BTV passive surveillance during December 2008 in Bregenz following the identification of the first BT case in that district. Additionally, AGES provided a shape with information on domestic cattle and sheep numbers per parish and parish areas in Austria. Maps of *Culicoides* abundance and risk areas for BT occurrence in Austria, for spring, summer and fall seasons (from the work of Silbermayr, 2009) were also used in this chapter.

4.1.2 Software tools and analytical methods

The georeferencing of the official German outbreak map was performed with gvSIG 1.1.9 and all the maps presented in this chapter were made with the use ArcMap 9.2. The statistical computing was performed with the software R 2.8.1.

A field study was carried out with two Austrian districts (Bregenz and Schärding) using the results from serological surveillance (with c-ELISA and RT-PCR) that was carried out throughout the months of November and December 2008.

The goal of the hypothesis testing was to assess whether the proportion of c-ELISA positive samples which resulted positive for RT-PCR had a statistically significant association with the district of origin and hence with the presence of a mass vaccination campaign prior to the first case occurrence. The odds ratio (OR) was estimated to preview the strength of association in case a statistically significant association would be demonstrated.

In the hypothesis testing a two-sided test was used. Fisher's exact test was used as available data could not fulfill all the assumptions required for the use of Pearson's Chi-squared test, namely: i) assume that individuals were randomly selected from the population (Petrie & Watson, 1999); ii) all observations are independent; iii) the categories are mutually exclusive and exhaustive; iv) the expected frequency in any of the four cells of the table cannot be less than 1 and no more than 20% of the cells can have an expected value of less than 5 (Boslaugh & Watters, 2008). A significance level of 0.05 was adopted.

4.2 Results and discussion

The occurrence of BT in Austria in year 2008 took place in two different districts: 10 cases were identified in Schärding, between November 6th and December 17th, and one case was found in Bregenz, on December 10th. Schärding is located in the north eastern Austrian region of Upper Austria, on the border to the south eastern German region of Bavaria, while Bregenz is on the western region of Vorarlberg, on the border to the German region of Baden-Wurttemberg.

Before the first case in Schärding was reported there was no ongoing vaccination in that district (BMGFJ, 2009). Unfortunately, there was no information available concerning the proportion of domestic ruminants that were already vaccinated in Bregenz by the beginning of November 2008, although it is certain that the vaccination has started in this district on August 2008 (BMGFJ, 2009).

In both districts, appropriate conditions for BTV-8 spread and establishing were analyzed, as reported below, and considered nearly equivalent, except for the presence of vaccinated ruminants.

4.2.1 Epidemiological equivalence between districts

Even though Bregenz and Schärding are located in different regions, both are in the Austrian border to southern German regions where BTV-8 occurrence had been observed and where a vaccination campaign was ongoing by November 2008 (BMELV, 2008).

Due to a lack of available accurate geographical data regarding the BT cases occurred in Germany, Figure 15 was built out of an approximate georeferencing of the map included in the report submitted in November 2008, the closest dated to Schärding's first case occurrence, to SCFCAH by the Federal Republic of Germany (BMELV, 2008). The cases that are pictured inside of the southern German regions of Bavaria and Baden-Wurttemberg are the total number of BTV-8 positive samples (including old cases), from January 2008 to 10th November 2008. It's important to mention that there was no difference in the number of BT cases in the southernmost regions of Germany between the reports submitted in October, November and December 2008. However, one cannot discard the possibility that BTV-8 was still circulating in that region even tough there was no detection of new cases. Figure 15 shows the perimeter around the cases located closer to the Austrian border formed by both 100 km protection zones and 150 km restricted zones in accordance to the Council Directive 2000/75/EC.

Figure 15 – BT case density in Germany and perimeters of PZ and RZ around cases closer to the border with Austria.

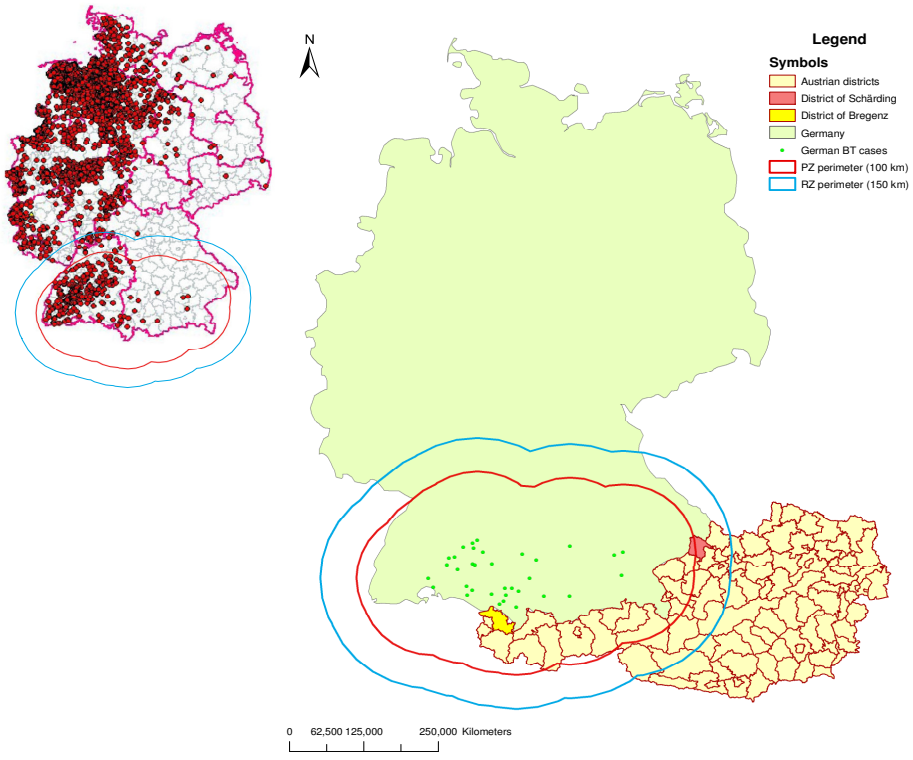


Figure 15 shows that both Schärding and Bregenz were situated inside of the restricted zones around the German cases identified closer to the border. It can also be observed that the case density in the German region Baden-Wurttemberg (north to Bregenz) was to a great extent higher than the case density in Bavaria (north to Schärding). In fact, Baden-Wurttemberg is among the identified risk zones in Germany for the optimal development and competence of *Culicoides* spp. to transmit BTV, contrarily to Bavaria (Koslowsky, Staubach, Kramer & Wieler, 2004). Even tough this fact does not prove epidemiological equivalence between the two Austrian districts, if it had an influence on BTV-8 establishing it would indicate Bregenz has a more risky district and not the opposite.

According to EFSA report on BTV-8 epidemic, the infection was spread into new areas predominantly in a horizontal east-west direction, and had a limited south spread and a very limited north spread. Density of wind events is so far the only variable identified which describes this spread pattern (EFSA, 2007a). If wind was to be considered the causal factor for BTV-8 arrival in Austria, many plausible possibilities could be considered: i) a southward spread from Bavaria to Schärding and a later southward spread from Baden-Wurttemberg to Bregenz; ii) a southward spread from Bavaria to Schärding with a further east-west spread from Schärding to Bregenz; iii) a southward spread from Bavaria to Schärding and a further south east-west spread from Bavaria to Bregenz. Unfortunately, due to time constraints it was not possible to perform a wind density model in order to assess the relationship between the BT cases in Austria and the vector spread by wind.

In order to investigate the conditions for within-herd spread in both districts, cattle and sheep densities were analyzed. Figure 16 and Figure 17 were built to illustrate a gradient of the cattle and sheep densities, together with the location of the BT cases occurred in both districts. It was observed that cattle and sheep densities were identical in the BT case location in Bregenz comparing to case locations in Schärding. More specifically, all cases were located in parishes with high cattle and low sheep densities. However, the parish in the district of Bregenz where BTV-8 was identified is surrounded by low-cattle density areas, whereas the affected parishes in Schärding are included in a wider area of high-cattle density. Therefore, the hypothesis that higher densities of cattle in the district of Schärding may have contributed to a faster spread of BTV-8 cannot be discarded.

Figure 16 – Cattle density in Austria and geographical location of BT cases occurred in 2008.

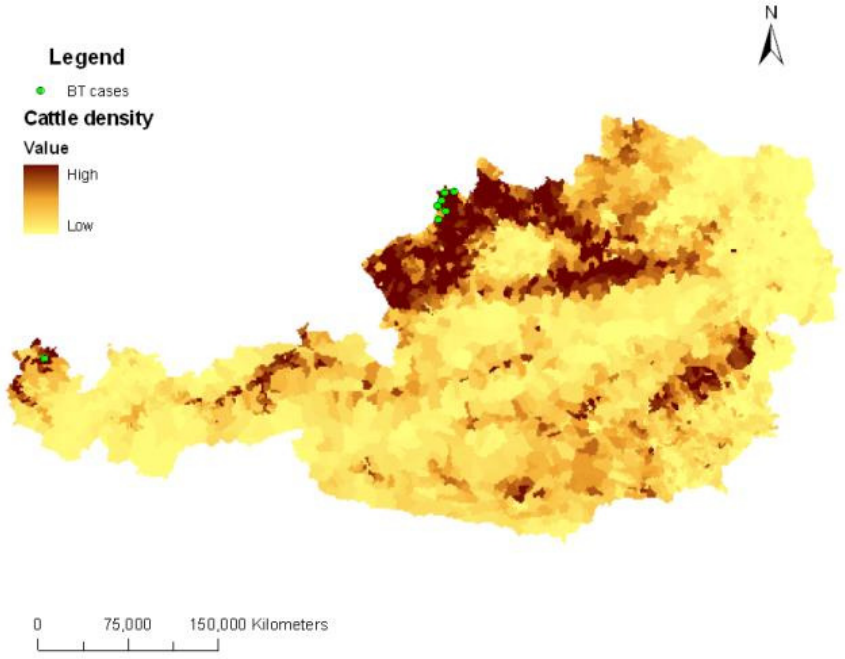
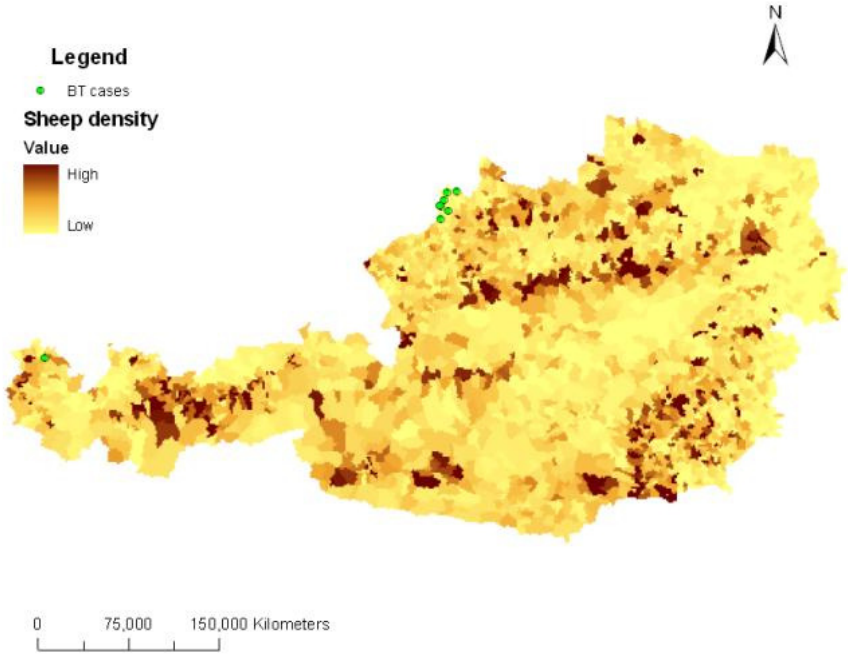


Figure 17 – Sheep density in Austria and geographical location of BT cases occurred in 2008.

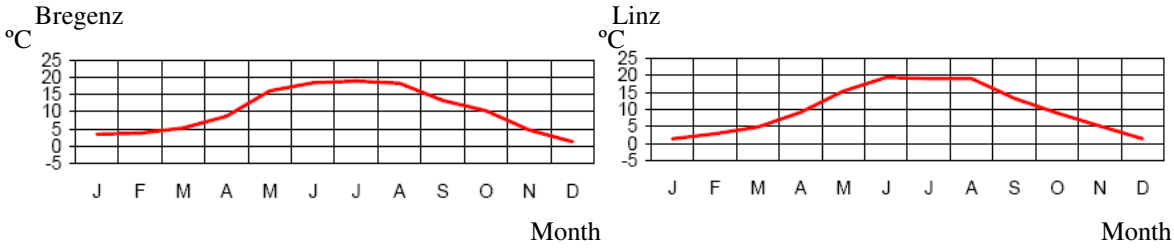


Wild ruminants may also play an important role in BTV-8 transmission. Therefore, estimating their number in each district is relevant. Schärding has an average of about 500 fenced wild ruminants, which are around 250, averagely, in Bregenz.

Competent vectors for BTV-8 have been identified in all traps set up late in 2007, including in both districts under analysis. The Obsoletus complex was prevailing in both locations (Sehnal et al., 2008).

The average monthly temperatures of Bregenz and Linz (the regional capital of Upper Austria, where Schärding is located) were analyzed for the year 2008 (Figure 18).

Figure 18 – Average monthly temperatures in Bregenz and Linz, in 2008.



Source of charts: AGES.

It was observed that the maximum temperatures reached in each district were both very close to the same value (20 ºC), and occurred in the same month (July). Furthermore, the raise of temperatures in spring was apparently very similar in both locations, starting in March and raising close to 5 ºC every month until reaching the peak in July. It can be assumed that temperature influence alone on virus transmission is therefore comparable between Schärding and Bregenz. However, humidity and rainfall influences were not possible to investigate; hence the effect of temperature alone cannot be taken as decisive and must be considered with care.

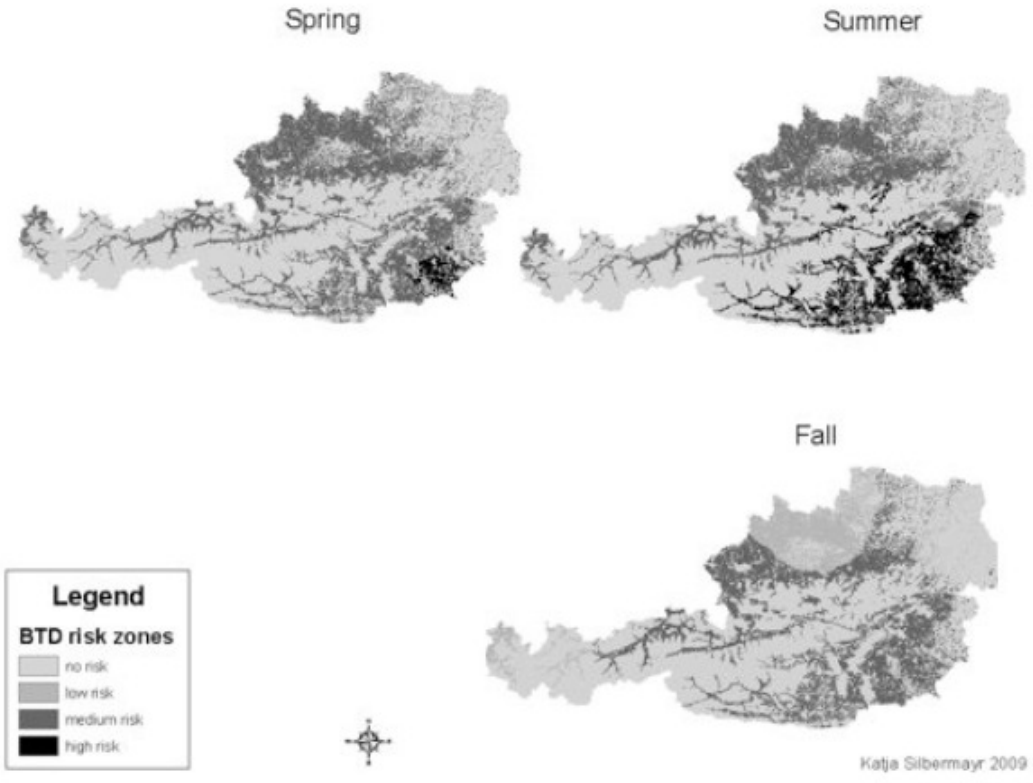
Mountains have been considered as a limiting landscape element for the spread of BT disease and a limit of 650 m was identified during the 2006 BTV-8 epidemic in northern Europe (EFSA, 2007a). Sehnal et al. (2008) indicates altitudes of 316 m in Schärding and 402 m in Bregenz, both under the above mentioned limit.

The control measures applied in Bregenz and Schärding differed in one crucial step – preventive mass vaccination. Bregenz is part of the Vorarlberg region. When this region was included in the PZ around German outbreaks, a vaccination campaign was immediately implemented, in August 2008, targeting all domestic ruminants in that region. In Schärding however, control measures were limited to movement restrictions and vector control actions, likely because this district was not totally included in the protection zone of the German

outbreaks, because case density in Bavaria was not as high as in Baden-Wurttemberg and/or due to a lack of available BTV-8 vaccines.

Sylbermayr (2009) developed a spatial risk assessment of bluetongue disease in Austria, identifying areas of high risk for hosting a potential BT outbreak. *Culicoides* surveillance data was collected in farms following minimum criteria: i) livestock farmstead holding more than 10 domestic ruminants; ii) close proximity to water bodies; iii) moderate altitudes (between 116 m and 1190 m); and iv) preferably farmsteads without Alpine transhumance. Climate data from 186 meteorological stations was also used in the analysis, specifically regarding monthly averages of i) absolute minimal daily temperature, ii) mean daily temperature, iii) relative humidity and iv) mean daily precipitation. Additionally, altitude of blacklight traps was also considered. *Culicoides* spp. distribution maps were created having in consideration the above mentioned variables, and were further combined with areas of domestic ruminant host contact. The result of this work was the creation of risk maps for BT occurrence for spring, summer and fall seasons in Austria, which are illustrated inFigure 18.

Figure 19 – Risk areas for BT occurrence in spring, summer and fall, in Austria.



Source of maps: Sylbermayr, 2009.

As can be seen in Figure 19, according to Silbermayr (2009), the districts of Schärding and Bregenz are included in areas with similar risk of BT occurrence, which varies from medium risk in spring and summer to low risk in fall.

In conclusion, even considering the innumerous factors that could not be included in the analysis of both districts, it can be reasonably assumed that Bregenz and Schärding owned very similar conditions for the establishment and spread of BTV-8 in the last months of 2008. The exception was the performance of a vaccination campaign that was in place in Bregenz since August 2008 comparing with the total absence of BTV vaccination in Schärding by the time of the first BT case occurrence, in November 2008. The implementation of preventive measures, as the application of vaccines in high risk non-infected areas, has the objective of reducing the probability of introduction and spread of diseases.

4.2.2 Hypothesis testing

A two-sided Fisher's exact test was performed to access the association between the proportion of PCR-positive (PCR_+) results in a c-ELISA positive sample, with the district of origin of that sample.

Between November 6th and December 30th 2008, 463 cattle sera from Schärding were scanned for the presence of BTV neutralizing antibodies with c-ELISA in the frame of BTV-8 active surveillance. Twenty nine samples tested positive.

In Bregenz, 36 cattle sera were tested with c-ELISA after December 10th. The samples were collected under the scope of a passive surveillance system, after the first confirmation of a BT case in the district, from animals suspicious of having had contact with the case. Thirty two out of the 36 samples tested positive.

All samples which resulted positive to c-ELISA were further tested with RT-PCR.

A contingency table was built (Table 6), with the results from samples which were tested with RT-PCR for the presence of BTV-8, after a positive result with c-ELISA. The district of origin was considered as the risk factor under analysis to which PCR_+ and PCR–negative animals have been exposed in the past.

Table 6 – Contingency table for comparison of PCR results between Schärding and Bregenz

District	PCR result		
	+	-	
Schärding	10	19	29
Bregenz	1	31	32
	11	50	61

To confirm if a statistically significant association in fact existed between the district of origin and PCR_+ results, a two-sided Fisher's exact test was performed based on Table 6. The null hypothesis (H_0) was that the proportion of PCR_+ in samples positive to c-ELISA (p) did not

depend on the district of origin (H_0: $p_{Schärding} = p_{Bregenz}$) whereas the alternative hypothesis (H_A) was that p depended on the district of origin (H_A: $p_{Schärding} \neq p_{Bregenz}$). The outcome of the test was a p-value of 0.001924. This result is statistically significant at a 5% level, which allowed the rejection of H_0 and the acceptance of H_A, meaning that there was a statistically significant association between the district of origin and the proportion of PCR_+ results in a c-ELISA positive sample.

The hypothesis testing was computed with the software R 2.8.1, and the commands are included in Annex I.

In order to measure the magnitude of the statistical association between the district variable and a PCR_+ result, the OR was estimated as a measure of strength of association.

$$odds_{Schärding} = \frac{10}{19} \approx 0.5263$$

$$odds_{Bregenz} = \frac{1}{31} \approx 0.0323$$

$$OR = \frac{0.5263}{0.0323} \approx 16$$

The result is to be interpreted as the odds of having PCR_+ results in Schärding is approximately 16 times the odds of having PCR_+ results in Bregenz, in samples which were positive to c-ELISA. The estimated 95% confidence interval for the OR was [1.96 ; 727.40], demonstrating a poor accuracy. Therefore, the above interpretation looses its significance. However, the most important to be noticed is that the lower bound of the confidence interval is greater than 1. For an OR greater than 1, the likelihood that the exposure to the supposed risk factor is associated with risk of disease increases, and the greater the departure from 1, the stronger the potential cause-effect relationship (Pfeiffer, 2002). In this case, OR indicates that there might have been an association between the district of Schärding and a higher proportion of PCR_+ results amongst c-ELISA positive samples.

Summarizing, the most plausible interpretation of the higher proportion of PCR_+ in the c-ELISA positive sera from Schärding compared to the c-ELISA positive sera from Bregenz is that the sera from Bregenz were most likely collected from vaccinated non-infected cattle. c-ELISA does not differentiate vaccinated from natural infected animals, whereas RT-PCR does; therefore c-ELISA positive results in Bregenz were most likely due to vaccine induced circulating antibodies. The c-ELISA positive sera from Schärding were partially due to natural infection with BTV (further confirmed with RT-PCR positive results) and in part due to lack of specificity of the c-ELISA which originated false-positive results. It has been shown

that c-ELISA tests have high sensitivity and not so high specificity (Shringi & Shringi, 2005), which corroborates the above interpretation of the results.

It must be emphasized that the data that was used for this analysis probably did not illustrate the true infection situation in the study populations. Previous studies have shown that RT-PCR detects BTV infections in an earlier stage (between days 2 and 5 p.i.) when compared to c-ELISA assays (day 7 p.i.). Since the first testing for BTV surveillance was performed with c-ELISA and only the positive results were further confirmed with RT-PCR, even tough c-ELISA has a high sensitivity, the possibility that false negative results could have been ignored must still be considered. This possibility, once confirmed, has contributed to the maintenance of infected animals in the field, with all the resulting consequences in terms of BTV establishment and spread.

Chapter 5: Evaluation of the cattle population dynamics in Styria and its influence on the duration of vaccination immunity

The region of Styria was considered in a great extent as a high risk zone for BT occurrence (Silbermayr, 2009), although no cases were reported to date. An analysis was performed on the dynamic of the cattle population in that region in order to evaluate if the lost of herd immunity threshold is mostly due to population changes or otherwise, if it will depend mainly on the duration of the immunity conferred by vaccination.

5.1 Material and methods

5.1.1 Data sources

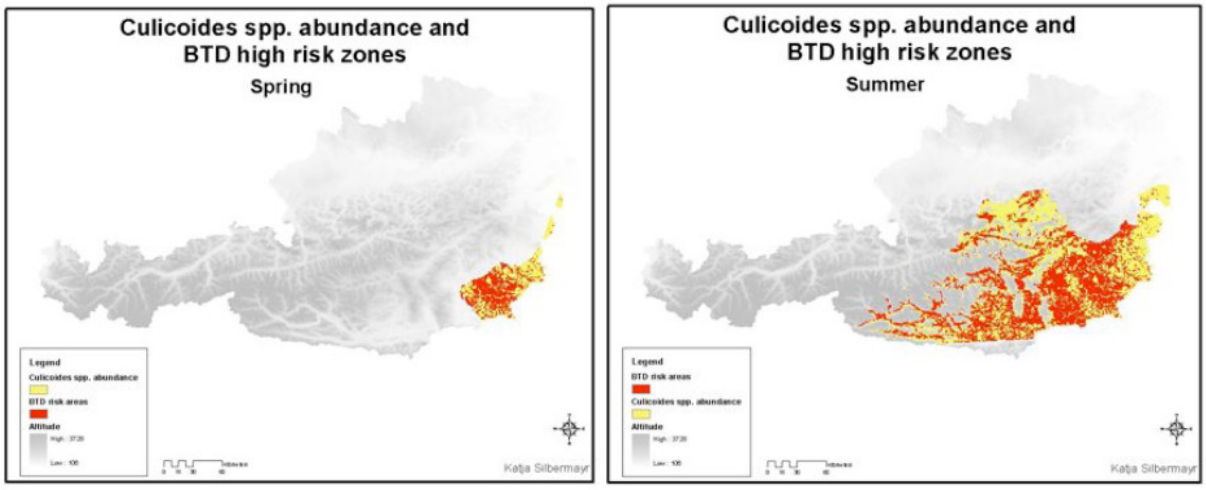
In order to assess the dynamic of the cattle population in the region of Styria, the following estimates and data were needed: i) to estimate the number of cattle in that region using a dataset with the number of cattle per district in Austria; ii) to estimate birth and death monthly numbers from a dataset which indicated the dates of birth, natural death (death at the farm) and slaughtering of 24,485 cattle individuals in Styria, between November 2007 and October 2008; iii) to estimate the monthly numbers of imports and exports to and from Styria, respectively based on cattle movements between Styria and the remaining Austrian regions, relating the period from September 2008 to August 2009. Data on cattle movements between Styria and foreign countries could not be acceded for the analysis.

5.1.2 Software tools and analytical methods

The data management and the statistical computing were made using the software packages of Microsoft Office, Excel 2003 and Access 2003.

Styria is the south eastern region of Austria. Although no BT occurrences have been recorded in this region to date, it apparently owns suitable conditions for vector's abundance and BTV infection. Silbermayr (2009) built risk maps for bluetongue disease occurrence in Austria, and on both seasons which posed higher risk (spring and summer), Styria comprised the majority of the area at risk (Figure 20).

Figure 20 – Risk maps for *Culicoides* spp. abundance and BT occurrence in Austria, on spring and summer seasons.



Source of maps: Silbermayr, 2009; BTD means bluetongue disease;

The mountainous barrier represented by the Alps, which cross the country separating the BTV-8 affected regions of Upper Austria and Vorarlberg from Styria, might be a plausible reason for the absence of BT cases in this region as far as virus spread by mean of infected vector dispersion is concerned. However, movement of infected ruminants or ruminant live products also play an important role on BTV spread, thus the onset of BTV-8 in Styria in a coming future is a possibility which cannot be discarded.

Births, animal losses and animal movements are responsible for the variation of groups in a population. Following a mass vaccination campaign, the vaccinated animals constitute the immune sub-population whereas non-vaccinated animals represent the susceptible group, provided that immunization does not occur by other means, and assuming that the totality of vaccinated animals are effectively immunized.

The BT vaccination campaign started in Styria in December 2008 and ended by March 2009. Data on the proportion of cattle that had been vaccinated until the end of March was not accessible for the analysis. However, it is known that the campaign excluded cattle younger than 3 months old, hence born after December 2008 (BMGFJ, 2009).

Immunity against BTV-8 can be conferred either by vaccination, ingestion of colostrum from a vaccinated cow or by natural infection with the virus. No evidence of virus circulation was found in the region of Styria to date, being therefore assumed that natural infection is unlikely to occur in this region. The non-vaccinated young animals could have been passively immunized with colostrum from vaccinated cows. However, colostral immunity has been described to not last more than 39 days of age in calves (EFSA, 2007d). Thus, this group of animals became successively susceptible to BTV between June and September 2009.

Summarizing, non-vaccinated young cattle, in Styria's case, are the fraction of the population that can be considered to be part of the susceptible sub-population after the end of the vaccination campaign.

The monthly numbers of births, deaths, slaughters, imports and exports of cattle were estimated. Their proportions, compared to the average number of cattle in Styria, were calculated. The interrelation between these population parameters reflects the monthly variations in Styria's cattle population. The estimated rates of birth and import were summed up to constitute the monthly ingress rate whereas slaughter, death and export rates were summed up to calculate the monthly exit rate. The difference between monthly exit and ingress rates was estimated and the sum of these estimates resulted in the average population proportion varying in a whole year period.

5.2 Results and discussion

The monthly numbers and proportions of cattle births, deaths and slaughters in Styria, are presented in Table 7.

Table 7 – Monthly numbers and proportions of cattle births, slaughters and deaths in Styria.

Year	Month	Births	Slaughters	Natural deaths	**Average cattle population:** 336825		
					Births proportion	**Slaughters proportion**	**N. deaths proportion**
2007	November	2	0	0	0.000006	0.000000	0.000000
	December	1	0	0	0.000003	0.000000	0.000000
2008	January	5	0	2	0.000015	0.000000	0.000006
	February	4	0	0	0.000012	0.000000	0.000000
	March	6	0	0	0.000018	0.000000	0.000000
	April	19	0	0	0.000056	0.000000	0.000000
	May	10	0	1	0.000030	0.000000	0.000003
	June	12	0	4	0.000036	0.000000	0.000012
	July	13	0	3	0.000039	0.000000	0.000009
	August	22	3	2	0.000065	0.000009	0.000006
	September	2596	256	268	0.007707	0.000760	0.000796
	October	7168	641	784	0.021281	0.001903	0.002328

The monthly numbers and proportions of imports and exports between Styria and the remaining Austrian regions are shown in Table 8.

Table 8 – Monthly numbers and proportions of cattle imports and exports, in Styria.

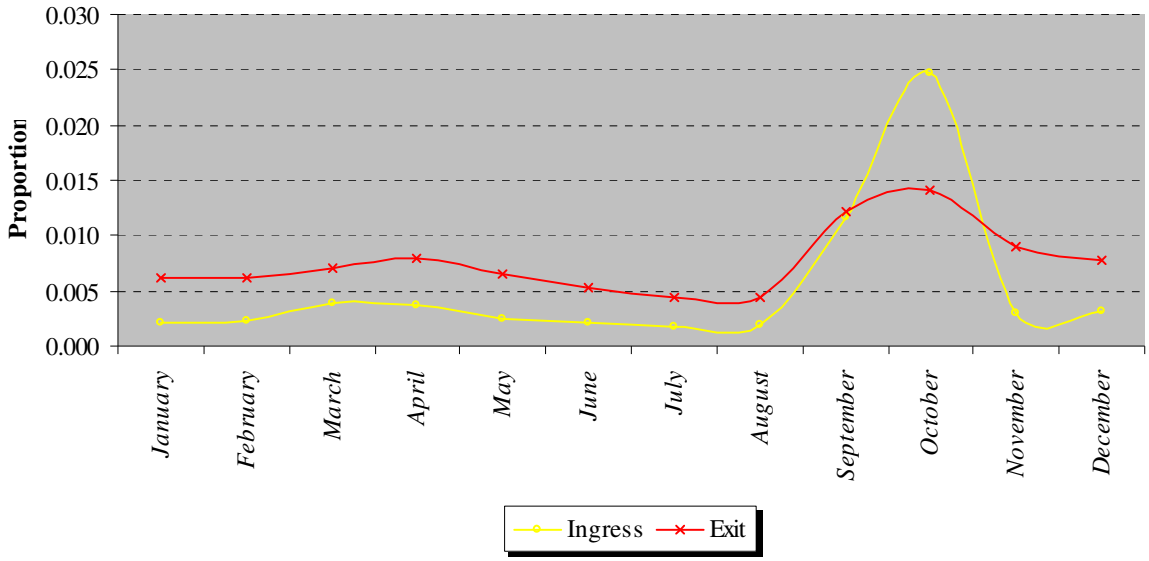
Year	Month	Imports	Exports	**Average cattle population:** 336825	
				Imports proportion	**Exports proportion**
2008	September	1345	3605	0.003993	0.010703
	October	1169	3306	0.003471	0.009815
	November	993	3007	0.002948	0.008927
	December	1085	2589	0.003221	0.007686
2009	January	698	2072	0.002072	0.006152
	February	778	2078	0.002310	0.006169
	March	1288	2377	0.003824	0.007057
	April	1226	2701	0.003640	0.008019
	May	842	2204	0.002500	0.006543
	June	721	1800	0.002141	0.005344
	July	603	1464	0.001790	0.004346
	August	644	1490	0.001912	0.004424

The data that was available for the present analysis did not allow for the estimation of all rates for the same time period. More specifically, it was only possible to have access to monthly numbers of cattle births, deaths and slaughters respecting the period between November 2007 and October 2008 and to monthly numbers of imports and exports between Styria and the remaining Austrian regions, from September 2008 to August 2009. Nevertheless, it was assumed that those estimates showed in Table 7 and Table 8, providing they illustrate the reality, do not vary significantly from one year to the next.

Moreover, data concerning births, deaths and slaughters showed an expressive aggregation in the months of September and October, which could not be confirmed to correspond to the reality of that region. Furthermore, it was not possible to include data about imports and exports between Styria and foreign countries in the analysis, which could make a difference in the overall change of the cattle population.

Despite of the above mentioned data limitations, the estimated rates of birth and import were summed to constitute the monthly ingress rates; slaughter, death and export rates were summed to form the monthly exit rates. Ingress rate represents the entering of new animals in Styria's cattle population every month, whereas exit rate represents the exit of individuals from that population. The dynamics of the estimated cattle ingress and exit rates along the year is illustrated in Figure 21.

Figure 21 – Dynamics of cattle ingress and exit rates for Styria.



According to these numbers the average monthly difference between the number of cattle exiting the population (by slaughter, natural death or export) and the number of new cattle entering the population (by birth or import) is 0.2%. After one year it is estimated that the population has varied in a proportion of 2.8%.

A year variation around 3% is not sufficiently high to produce significant changes in the immune and susceptible sub-populations in the time-frame of one year. The lost of population immunity will therefore be mainly due to the end of protection conferred by the inactivated vaccine. This protection was estimated to last around one year (Hamers et al., 2009). Considering the period when vaccination was performed in Styria, one can assume that Styria's cattle population will have lost its BTV immunity, the latest by the end of March 2010, given that no revaccination plann will be in place earlier.

Chapter 6: BTV transmission model for the region of Styria

In this chapter, a transmission model for BTV in Styria was developed. In chapter 5 it was observed that the lost of population immunity in Styria will depend mostly on the end of immunity conferred by vaccination. Therefore, it is important to assess the risk of BT occurrence in a cattle population that, in the absence of a revaccination plan in 2010, will become again susceptible to BTV.

6.1 Material and methods

6.1.1 Data sources

In order to estimate monthly basic reproduction numbers for Styria, the following data was used: i) information on *Culicoides* abundance in Styria, deriving from entomological data collected in 9 sampling sites across that region between April 2008 and February 2009; ii) local climate data, specifically average temperatures associated to each entomological sampling site in Styria for the above mentioned months; iii) a dataset with the average number of domestic cattle per herd at the entomological sampling sites in Styria; iv) a dataset which indicates the dates of death of 24485 cattle individuals in Styria between November 5th 2007 and October 31st 2008.

6.1.2 Software tools and analytical methods

The data management and statistical modeling were performed with the software package of Microsoft Office Excel 2003 and with @RISK.

"The basic reproduction number (R_0) provides a powerful tool when assessing the risk of disease invasion" (Gubbins et al., 2008). It is defined as the average number of secondary infections deriving from the introduction of a single infected individual in a naïve population (100% susceptible individuals) (Wonham, Beck & Lewis, 2004; Heffernan et al., 2005; Smith, McKenzie, Snow & Hay, 2007; EFSA, 2007d; Racloz et al., 2008; Gubbins et al., 2008). Alternatively, "the fraction of a population that would need to be protected to confer "herd immunity" and interrupt transmission is $1\text{-}1/R_0$" (Smith et al., 2007). Therefore, R_0 provides an index of transmission intensity and establishes threshold criteria. Gubbins et al. (2008) assessed the risk of BT to United Kingdom livestock by computing R_0 for BTV with a temperature-dependent model. Similarly, Racloz et al. (2008) computed monthly R_0 values for BT occurrence in Switzerland using the equation described by EFSA (EFSA, 2007d) and previously used by several authors who estimated R_0 for other vector-borne diseases, such as for Malaria (Smith el al., 2007) or for West-Nile virus (Wonham et al., 2004).

In the present study, a BTV transmission model for domestic cattle of Styria was developed by computing R_0 monthly values for BT infection using entomological and climate data from that region, between April 2008 and February 2009.

The input parameters used to compute R_0 values are described in Table 9 and were based on available scientific data and field data from the region of Styria.

Table 9 – Input parameters for the BTV transmission model.

Notation	Parameter	Value or range	Reference
Hosts			
λ	cattle death rate or removal rate per day (not affected by disease status)	0 – 0.0008	Monthly field data from Styria
i	rate at which infected hosts become infectious (in days)	1/3	EFSA (2007d)
$1/i$	incubation period in the host (in days)	3	EFSA (2007d)
v	duration of viraemia in infected hosts (in days)	21	Bonneau et al. (2002)
r	recovery rate per day (rate at which infectious hosts become immune)	1/21	EFSA (2007d)
Vectors			
a	biting rate per day	$0.0002t(t\text{-}3.7)(41.9\text{-}t)^{1/2.7}$	Gubbins et al. (2008)
b_1	probability of transmission from host to vector	0.15	Gubbins et al. (2008)
b_2	probability of transmission from vector to host	0.9	EFSA (2007d); Gubbins et al. (2008)
τ	extrinsic incubation period (in days) (required for the virus replication and colonization of salivary gland cells)	10	EFSA (2007d)
μ	midges mortality rate (per day)	0.0667	EFSA (2007d)
$e^{-\mu\tau}$	proportion of infectious midges which survive the extrinsic incubation period	$2.718^{-0.0667(10)}$	EFSA (2007d)
t	average daily temperature (ºC)	9 -19	Monthly field data from Styria
m	vector-to-cattle ratio (per day)	4 -177	Monthly field data from Styria

The estimation of R_0 for vector-borne diseases needs to account for the biology of the host, the disease agent and the vector. The equation described by EFSA (2007d) to compute R_0 for BT, takes into account parameters which reflect those three components of the disease, and was therefore selected to be used in the present model.

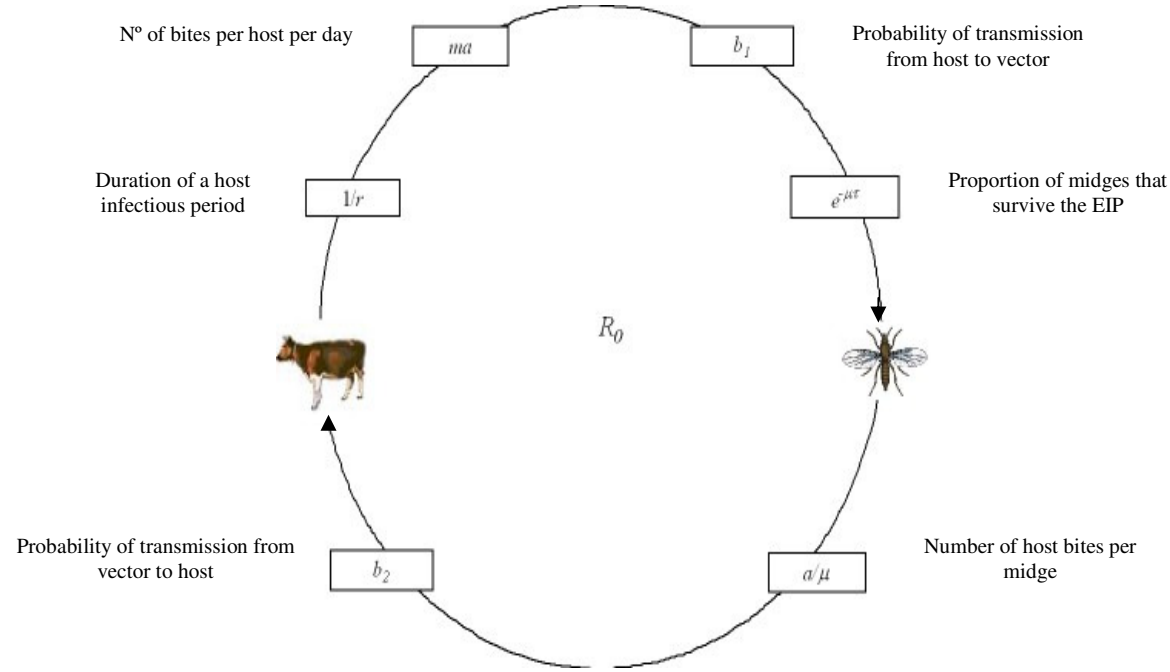
According to this model, the BTV life cycle can be described as follows:

- The rate of infected hosts becoming infectious per unit of time is i (due to an incubation period of $1/i$);
- infectious hosts recover and become immune at a rate r (recovery rate), which corresponds to 1/viraemia ($1/v$);
- Death of hosts (in this case, specifically of cattle), independently of their disease status, occur at a rate λ;
- Among the vectors population, susceptible midges bite viraemic hosts at a rate a, although just a proportion b_1 of the bites lead to infection (probability of transmission from host to vector);
- A proportion $e^{-\mu\tau}$ of the newly infected midges become infectious to hosts if they survive the EIP (τ), assuming a vector death rate of μ;
- A proportion b_2 of bites from an infectious midge infects a new host (probability of transmission from vector to host);

Ultimately, a susceptible host becomes infected at a certain infection rate, depending on a, b_2, the number of infectious *Culicoides* and the vector-to-host ratio (m). According to Gubbins et al. (2008), working with vector-to-host ratios is more convenient than to work with host and vector population sizes.

Figure 22 illustrates the above described BTV life cycle model. The infected host remains infectious for a $1/r$ period, the midge bites a host ma times per day and a b_1 proportion of bites on an infected host infect a midge. An $e^{-\mu\tau}$ proportion of midges survive the EIP and bite hosts at an a/μ rate. A b_2 proportion of these bites infect new hosts.

Figure 22 – BTV life cycle model



Adapted from EFSA (2007d).

The present model was based on a deterministic approach (EFSA, 2007d; Racloz et al., 2008). However, some updates had been made to the reference model:

- The influence of temperature on determining R_0 for BT occurrence is most likely due to the number of temperature dependent processes involved in the transmission of BTV, namely the biting rate, the vector mortality rate and the EIP (Gubbins et al., 2008). For the parameter of vector biting (*a*) rate, temperature dependence was incorporated, as described by Gubbins et al. (2008). Unfortunately, the information available on scientific literature was not sufficient to introduce temperature dependence on the parameter EIP. Temperature dependence was included in the variable of vector mortality rate (μ), as described by Gubbins et al. (2008), but the result was not conclusive. Temperature-dependent μ determines the probability that a vector will survive the EIP ($e^{-\mu\tau}$), which greatly influences the R_0 value. Altough, the results showed that higher vector mortality rates matched higher values of R_0. It was decided therefore not to incorporate temperature dependence in the parameter and to use the value described by EFSA (2007d).

- Some input parameters could be estimated with field data from Styria, instead of being adopted from literature, namely: i) daily cattle death rates (λ) were estimated from data of domestic cattle death numbers in Styria between November 5th 2007 and October 31st 2008 and assumed to vary at a negligible rate between different years; ii) the used average temperatures (*t*) for Styria were obtained from *Culicoides* sampling sites in this region, and iii) vector-to-host monthly ratios (*m*) were estimated as the ratio of the number of

Culicoides spp. monthly catches in Styria to the average number of cattle individuals in farms where the traps had been set.

- The value of the probability of transmission from host to vector (b_1) used in the transmission model for BTV described by EFSA (2007d) was 1%. However, Gubbins et al. (2008) used for the same input variable a range between 0.1 and 15%. In fact, it has been described that oral susceptibility of *Culicoides obsoletus,* the prevailing vector species in Styria, to BTV may vary between different geographic regions (Carpenter, Lunt, Arav, Venter & Mellor, 2006). Therefore, since there is no information on the values of oral susceptibility of *C. obsoletus* to BTV in Styria, the value of 15% was adopted to develop the present transmission model, considering a worst case scenario.
- Bonneau et al. (2002) described the maximum duration of viraemia infectious to *Culicoides* as being 21 days. Therefore, this was the value adopted for the input variable duration of viraemia (v) for the present model.

The R_0 can be written as a product of four terms (EFSA, 2007d):

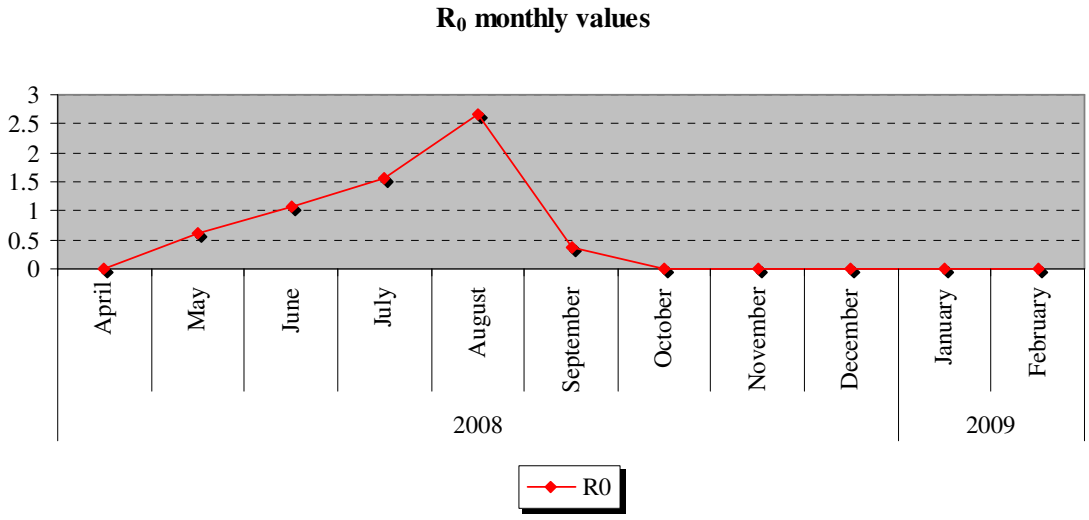
$$R_0 = \frac{i}{i+\lambda} \times \frac{mab_1}{(r+\lambda)} \times e^{-\mu\tau} \times \frac{ab_2}{\mu}$$

The first term is the probability that an individual host will survive during the intrinsic latent period and become infectious; the second term reflects the number of susceptible midges infected with BTV by an infectious individual host during its entire infective period; the third term is the probability that a midge will survive during the EIP and become infectious; the fourth term relates to the number of susceptible hosts infected with BTV by an infectious midge during its entire infective period.

6.2 Results and discussion

The estimated monthly transmission values (R_0) for the region of Styria, between April 2008 and February 2009, are summarized in Figure 23.

Figure 23 –Variation of the R_0 monthly values.



The highest R_0 estimates were associated with summer season months (July and August). The peak estimate, observed on August, was an R_0 of 2.66. This tendency is in concordance with the results obtained by Racloz et al. (2008); when the authors estimated monthly R_0 values for Switzerland for the years 2005 and 2006, peak values corresponded to the months of July and August in each year, respectively.

Gubbins et al. (2008) observed that values of R_0 higher than 1 were associated with higher values of the probability of transmission from host to vector (b_1), of vector-to-host ratio (m) and of temperature (t). In the present model, b_1 variable input was a fixed value; therefore, it was not possible to analyze the variation of R_0 against b_1. However, biting rate (a) obviously influences transmission from both host to vector and vector to host. In order to analyze the effect of a, m and t in the value of R_0, those variables were plotted along with the calculated monthly transmission values (Figure 24 to Figure 26).

Biting rate (a) showed to be a critical parameter for the transmission of BTV. Figure 24 shows that high biting rates were related with the peak values of R_0 on summer months.

Figure 26 shows that the R_0 values were higher when temperatures were between 15 ºC and 25 ºC, similarly to the previous findings of Gubbins et al. (2008) and Racloz et al. (2008). Furthermore, months with R_0 values lower than 1 (April to May 2008 and September 2008 to February 2009) registered average temperatures lower than 10 ºC, findings which are likely due to the fact that at lower temperatures BTV is unable to replicate to transmissible levels (Gubbins et al., 2008).

It was also observed that the variation of the vector-to-host ratio (m) matched to a great extent the variation of R_0 monthly values (Figure 25).

Figure 24 – Transmission value versus monthly biting rate (*a*).

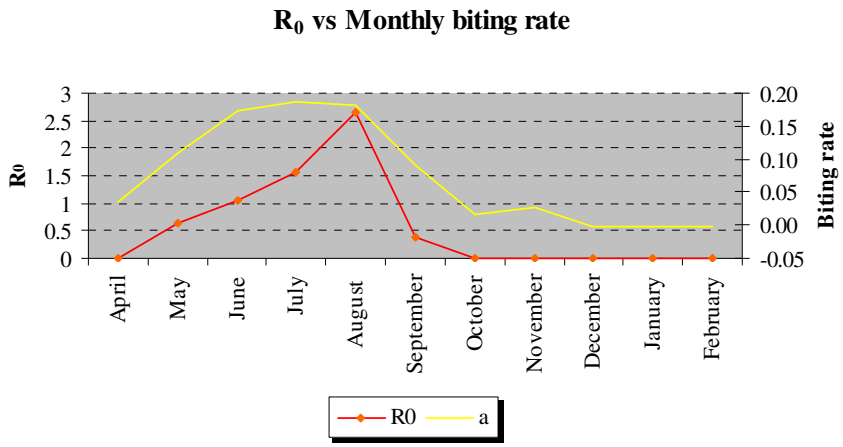


Figure 25 – Transmission value versus average monthly vector-to-host ratio (*m*).

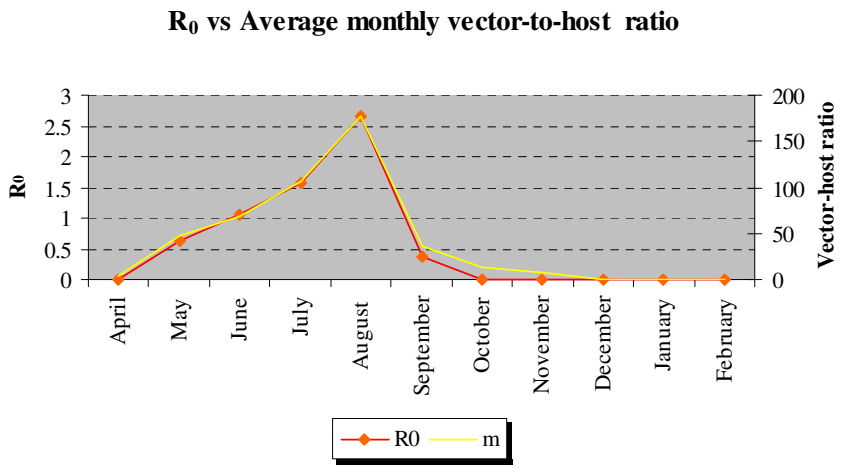
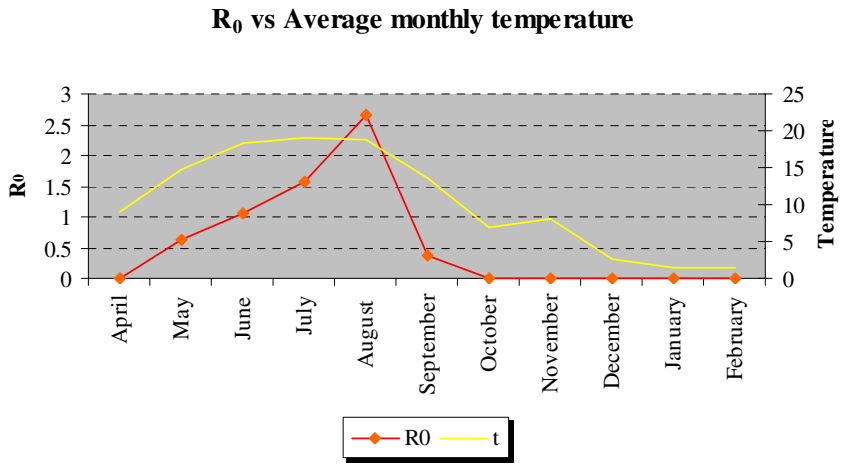


Figure 26 – Transmission value versus average monthly temperature (*t*).



These results corroborate those of Gubbins et al. (2008) in the stochastic transmission model for BTV in United Kingdom.

The computed R_0 values respect to the period between April 2008 and February 2009. Assuming that the field conditions (average monthly temperatures, vector to host ratios and average monthly death rates of cattle) will not vary significantly for the period between April 2010 and February 2011, the present model predicts that between June and August 2010, the risk of BTV transmission in Styria will exist, provided that a revaccination campaign will not be started in the meantime. If BTV is introduced in this region and the presence of at least one viraemic animal in the field is confirmed between June and August 2010, the risk of occurrence of secondary cases is not negligible, and can give rise to the first BT outbreak in Styria.

In order to control the spread and avoid the establishment of BTV, the R_0 should be reduced to less than one, which can be achieved by apllying a combination of several measures that will influence parameters used in the computing of the transmission number (EFSA, 2007d), specifically:

- Reduce the vector to host ratio (m) by larval control, animal housing (even though *C. obsoletus* has been described to be endophilic and endophagic, this measure may result preventing attack from other vector species) , repellants, insecticides and moving animals into free or low vector density areas;
- Reduce the duration of viraemia (v) by vaccination or by culling the infected animals;
- Increase the *Culicoides* mortality rate (μ) by using insecticides;
- Shorten the duration of stationing animals in an infected area ($1/\lambda$) (the parameter λ could also be interpreted as the daily removal rate and this measure can be interpreted as reducing the time period that an animal spends in an infected area during transport).

6.2.1 Model limitations

The present model is considered a *population level model*, since it assumes a homogeneous mixing of the population and is based on the principle of mass action, under which the rate of new infections is proportional to the current numbers of susceptible hosts and infectious midges in the considered population. Though, this assumption is far from the real transmission process of BTV in livestock populations. In reality, the rate of contact between *Culicoides* and hosts depends on their spatial distribution, thus, to be more accurate, the model should include spatial population structure. The model, as it is, can only be acceptable for a small geographical area where homogeneous mixing can be considered possible.

The natural transmission of BTV is also a seasonal phenomenon, depending on the climatic season. Different monthly average temperatures, the temperature dependence of biting rate and monthly estimates of vector-to-host ratio reflected to a certain extent the seasonal variation on the vector density and its interaction with the host. However, it must be noticed that vector counts resulted from trapping sites that are part of a surveillance warning system, therefore corresponding to high risk areas for *Culicoides* occurrence. As a consequence, monthly average numbers of *Culicoides* may have been over-estimated and may not reflect the reality of the whole region.

This study focused exclusively on BTV transmission to cattle. The estimates of vector-to-host ratio were obtained from cattle-only farms in Styria. However, as described by Gubbins et al. (2008), R_0 tends to be higher on cattle-only farms likely because of different vector preference and relative duration of viraemia between cattle and sheep. This indicates that there might have been an over-estimation of R_0 values for the region of Styria in the present transmission model.

Bonneau et al. (2002) described that BTV titres in the blood of infected ruminants vary during the viraemia period. In the present model however, host viral titre is assumed to be constant throughout viraemia, which may also have resulted in an over-estimation of R_0.

The conclusions of the present model are only valid for the parameters values considered. In order to extend it to other regions and/or time periods it would be necessary to adjust the parameters values, mainly the vector-related ones (biting rate, extrinsic incubation period, vector mortality rate, probability of transmission from host to vector and vector-to-host ratio), which tend to be less robust and to vary with species and climate (Gubbins et al., 2008).

Chapter 7: Conclusions

From the analysis of the BT cases occurred in Schärding it was concluded that the moments of infection were very likely between May and October 2008, considering the optimal temperatures for *Culicoides* abundance that were verified in the region between April and September. It could not be determined however if those cases were the result of BTV spread from previous cases occurred in southern Germany. The fact that the cases were reported in the context of an active surveillance system was the main reason why it was not possible to draw neither a temporal pattern nor a spatial pattern of the outbreak, which would both be useful to identify a possible source of the virus and the direction of its spread. These results confirm therefore that passive surveillance is most appropriate to be applied in the beginning of an epidemic before the disease presence is recognized.

The Austrian districts where BTV has been detected in late 2008, Schärding and Bregenz, even though located in different regions, where considered to have nearly equivalent conditions for BTV-8 establishment and spread. However, a higher cattle density in Schärding may have contributed to a higher spread of BTV, whereas the performance of a preventive mass vaccination campaign in Bregenz, most likely contributed for the opposite. It was not possible to accomplish the original goal of verifying the effect of preventive mass vaccination against BT. However, it was concluded that the proportion of PCR_+ results amongst c-ELISA positive sera was statistically associated to the district of origin. A much lower proportion was observed in Bregenz when compared to Schärding. This result was most likely due to the presence of vaccinated animals in Bregenz. It was concluded that distinct diagnostic techniques should be used in BTV surveillance, under distinct circumstances. In a vaccinated population it is expectable that the results of a serological surveillance will be mostly positive, unless a DIVA strategy is in place. Inactivated vaccines allow the implementation of DIVA. Until recently this was only possible with the use of RT-PCR techniques, however, alternative serological tests (i-ELISA) are being developed to allow an effective serological surveillance in a population immunized with inactivated vaccines. These tests should be the next recommended techniques to use in serological surveillance of vaccinated ruminant populations in Europe, where inactivated vaccines are the recommended and most used immunization techniques.

In the framework of the BTV active surveillance system that has been performed in Austria in 2008, c-ELISA negative results were not further tested with RT-PCR tests. Even tough c-ELISA assays are considered to have high sensitivity, the possibility of false-negatives occurrence can still not be discarded, since BTV infection in earlier stages (before 7 days p.i.)

is usually not detected in those assays. In order to keep the same surveillance approach, it is advisable to use a different serological test. Specifically, IgM-capture ELISA tests allow for the detection of BTV infections in earlier stages.

It has been suggested that factors reducing HIT may assist in the maintenance and spread of BT (Ward & Carpenter, 2007). The analysis of the dynamics of cattle population in Styria was performed under many data limitations. However, assuming that the 3% year variation in cattle numbers has in fact a negligible effect on the decrease of the HIT in a time-frame of one year, the lost of population immunity to BTV in Styria will be mostly due to the lost of immunity conferred by vaccination. This immunity has been estimated to last close to one year. Therefore, in the absence of a revaccination plan in 2009-2010, Styria will have become again susceptible to BTV by the end of March 2010. Additionally, it is still unknown whether or not immunization in one season affects the probability of infection with a homologous serotype in a subsequent season. Even tough a protective effect should exist, immunological variation within populations of BTV may be responsible for re-infection with homologous serotypes, even in the presence of substantial herd immunity (Ward & Carpenter, 2007). Therefore, Styria's cattle population may become susceptible to BTV-8 even earlier.

The results of the BT transmission model for Styria indicated that the risk of occurrence of secondary infections in the summer months is not negligible with an estimated maximum R_0 of 2.66. Summer season has been previously indicated by Silbermayr (2009) has a period of high risk of BT occurrence in Styria. Therefore, if a revaccination campaign against BTV will not be performed in the year 2010 and if the virus is introduced in that region before or throughout the summer season, a BT outbreak may in fact occur in Styria, in that year. It would be usefull to develop the present transmission model for all the regions in Austria, in order to assess and interpret different transmission values across the country, and wheter these estimates matched the field evidences of BTV establishment and spread. Furthermore, efforts should be maid to account for the effect of vaccination schemes in transmission of BTV.

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ANNEX I

R Commands

```
> contingency.table <- matrix (c(10,1,19,31), nrow=2, byrow=T)
> dimnames(contingency.table) <- list(c("PCR+","PCR-"),c("Schärding","Bregenz"))
> contingency.table

     Schärding Bregenz
PCR+        10       1
PCR-        19      31

> odds.schärding<- 10/19
> odds.bregenz<- 1/31
> odds.schärding

[1] 0.5263158

> odds.bregenz

[1] 0.03225806
>
> or<- odds.schärding/odds.bregenz
> or

[1] 16.31579

> fisher.test(contingency.table, alternative = "two.sided")

    Fisher's Exact Test for Count Data

data: contingency.table

p-value = 0.001924

alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:

  1.957997 727.395360

sample estimates:
odds ratio

 15.66011
```

ANNEX II

Training description

The first three months of my training period took place at the unit of Epidemiology and Risk Analysis of the Faculty of Veterinary Medicine of the Technical University of Lisbon. Dr. Telmo Nunes introduced me to the subjects of veterinary epidemiology, data management, descriptive statistics and risk analysis. Dr. Filipa Baptista introduced me to statistical modeling with the software SAS®. Eng Hugo Martins introduced me to the use of GIS in veterinary epidemiology, specifically to the use of the software packages ArcGis® and gvSIG.

The second three month training period was developed at the Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), in Graz, between 1st March to 28th May 2009 under the supervision of Prof. Dr. Klemens Fuchs, head of AGES division of Data Management, Statistics and Risk Assessment. During that period, I attended to the following courses:

- "Introduction to R" from 2nd to 12th March, by Dr. Johannes Hofrichter;
- "Introduction to Geographical Information Systems I", on 13th March, by M.S. Michael Schwarz;
- "Introduction to Geographical Information Systems II- practice with gvSIG" on 13th March, by MS Michael Schwarz;
- "Biostatistics I - Descriptive Statistics", on 17th April, by Prof. Dr. Klemens Fuchs;
- "Biostatistics I - Inference Statistics", on 8th May, by Prof. Dr. Klemens Fuchs.